

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2015366148 B2**

(54) Title
Preservation and transport of an ex vivo biological sample comprising ultrasound application

(51) International Patent Classification(s)
A01N 1/02 (2006.01)

(21) Application No: **2015366148**

(22) Date of Filing: **2015.12.18**

(87) WIPO No: **WO16/097390**

(30) Priority Data

(31) Number
14382548.7

(32) Date
2014.12.19

(33) Country
EP

(43) Publication Date: **2016.06.23**

(44) Accepted Journal Date: **2020.03.19**

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(56) Related Art
WO 2007143715 A2
US 6673594 B1



(51) International Patent Classification:
A01N 1/02 (2006.01)

(21) International Application Number:
PCT/EP2015/080688

(22) International Filing Date:
18 December 2015 (18.12.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
14382548.7 19 December 2014 (19.12.2014) EP

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: PRESERVATION AND TRANSPORT OF AN EX VIVO BIOLOGICAL SAMPLE COMPRISING ULTRASOUND APPLICATION

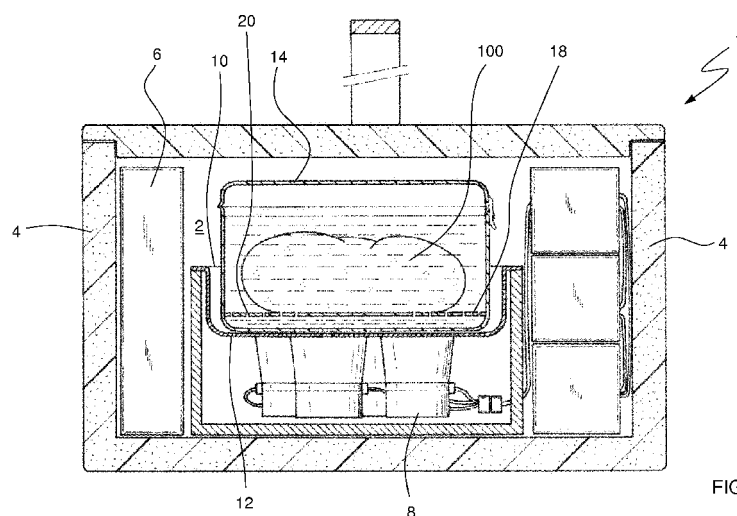


FIG. 7B

(57) Abstract: The present invention relates to a device for transport and preservation of an ex vivo biological sample and corresponding method. The device (1) comprises a chamber (2) for containing the biological sample (100), delimited by walls (4) made of a thermal insulating material. The device, furthermore, incorporates cooling means (6) that keep the temperature inside the chamber (2) below the temperature outside the device (1). Finally, an ultrasound system suitable for generating and applying ultrasound on the biological sample (100) is provided. The invention also proposes a method for transport and preservation which combines applying cooling and ultrasound to reduce cell damage in the biological sample.

PRESERVATION AND TRANSPORT OF AN EX VIVO BIOLOGICAL SAMPLE
COMPRISING ULTRASOUND APPLICATION

DESCRIPTION

Field of the invention

The invention relates to a device for transport and preservation of an *ex vivo* biological sample comprising a chamber for containing the biological sample, delimited by walls made of a thermal insulating material and cooling means for keeping the temperature inside the chamber below the temperature outside the device, as well as to a corresponding method for preservation of the biological sample.

The invention also relates to the use of the device according to the invention for transport and preservation of an *ex vivo* biological sample and subsequent transplant in a living human being or animal, or for subsequent laboratory research.

Background to the Invention

A reference herein to a patent document or any other matter identified as prior art, is not to be taken as an admission that the document or other matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

Where any or all of the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification (including the claims) they are to be interpreted as specifying the presence of the stated features, integers, steps or components, but not precluding the presence of one or more other features, integers, steps or components.

In the invention, the concept of "*ex vivo* biological sample" relates to an organ tissue that can be transported for subsequent transplant in a living human being animal, or for subsequent laboratory research.

5 For example, it is well known in the field of transplants that ischemia/reperfusion (I/R) syndrome is one of the main causes of both primary graft dysfunction (PGD) and primary graft failure (PGF), another transplantation being necessary in the latter case. Despite the advances achieved in surgical techniques in the past few decades, the injury the graft sustains during the period in which the donor organ is transported until it is implanted in the recipient (cold ischemia time) is still a major socially and clinically relevant problem yet to be solved. The incidence of PGF is 5- 10% and the incidence of PGD is 20-30% among transplanted organs. This all

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negatively results in a poor quality of life for the patient, the need for transplanting again, and more problems in the availability of organs for transplant.

There are different methods for storing a graft from the time it is removed from the donor until it is implanted in the recipient. In the case of dynamic normothermic preservation, *in vivo* conditions of the graft are imitated to the greatest extent possible throughout the entire preservation period. To that end, hypothermia is avoided, and the entrance of oxygen into the graft to prevent hypoxia and a reservoir for the exit of waste product are provided.

10

In static hypothermic preservation, which is the conventional preservation method, before the donor graft is transplanted in a recipient, the donor's organ or organs and tissues are subjected to an inherent ischemia time. Under such conditions, the method used for preserving the organ and tissues must comply with the following requirements: removing blood from the graft and rapidly cooling the graft by means of perfusion of the graft with the solution at 4°C, covering the graft with ice to assure cold conditions (2-6°C) and ischemic conditions. As a result, the injury the graft sustains during cold ischemia, i.e., due to a lack of blood supply and oxygen delivery under cold conditions, is reduced.

20

From the time the organ is removed from the donor, the graft is placed in a cooler, immersed in the preservation solution, and it is usually kept at 4°C until it is implanted in the recipient. The solution most widely used in the art is the University of Wisconsin solution, hereinafter UW solution.

25

The injury the graft sustains during transport in the cooler until it is implanted in the recipient (cold ischemia time, which is not usually less than 6 hours) is a key factor. This factor is responsible for PGD and PGF and negatively affects post-operative results, the quality of life and survival of the patient. It is known that the longer the time the organs remain in the cooler, the lower the viability of the graft that will be transplanted. The injury grafts sustain during the cold ischemia stage is the primary reason that a considerable number of organs (35%) cannot be considered suitable for transplant given their pathological conditions (kidney grafts from elderly, diabetic

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and/or hypertensive donors, fatty liver grafts, etc...). Such organs are extremely vulnerable to the injury they sustain during transport, before being implanted in the recipient, and have a higher risk of experiencing PGD and PGF. They are therefore not viable for transplant. These observations indicate that there is a need to find
5 alternatives for reducing the injury grafts sustain during cold ischemia before being implanted in the recipient.

Many of the components present in preservation fluids, the objective of which is to protect the graft under cold ischemia conditions in the cooler during the transport
10 thereof until it is implanted in the recipient, do not enter the graft and if they do, they do not reach the site of action at optimal concentrations in order to protect it. In fact, it has been found that when some of the components are removed from such preservation fluids, postoperative and post-transplant results are the same as if they were not removed.

15

Patent document WO 2007/143715 A2 relates to applying ultrasound to allow delivering oxygen to wounds and to aid wound healing. By way of example, the patent document very briefly discloses a device for preserving organs after they have been removed from the donor. To that end, the organ is placed in a vessel
20 which is supplied with a gas or liquid supersaturated with oxygen. It is a dynamic preservation system because the oxygen or liquid solutions saturated with oxygen contained therein gradually enter at controlled flow from an oxygen reservoir into the vessel, and waste products are gradually removed. The organ vessel is placed inside a semi-rigid container. The outer container further comprises an ultrasound
25 system for directing ultrasound on the container. There are no means for transmitting ultrasound between the inner vessel and the outer container, so the system cannot work as well as if the ultrasound was applied directly on a wound for the purpose of wound healing.

30 Studies indicating that application of ultrasound aids in delivering drugs and substances through tissues, such as the skin, and into the bloodstream, are also known. Nevertheless, in said studies ultrasound is applied *in vivo*, i.e., in the presence of blood flow. Patent document US 4,767,402 A, relating to the field of

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transdermal drug delivery by means of applying ultrasound, also relates to the risks associated with the use thereof. The patent document particularly advises against the prolonged use of ultrasound due to the risks associated with the increase in skin temperature and the possible damage this technique can cause. The same patent
5 document also discloses that ultrasound is poorly transmitted through air and that the preferred application thereof therefore requires a liquid medium. As a result, applying ultrasound for organ and tissue preservation is not advisable due to the mentioned factors of: local heating of the tissue, difficulty in penetrating considerable tissue or organ thicknesses, and transmission difficulty in gaseous media.

10

Patent document US 5,267,985 A discloses a method and apparatus for enhancing the diffusion of a substance to a local area of a material or tissue by means of ultrasound at two or more distinct frequencies. This patent document also discloses that the local temperature increase when ultrasound is applied is beneficial for
15 aiding in increasing penetration of the substances to be diffused.

The article (Sen Wang *et al.*, Medial hypothesis, 74, 2010; 147-149) suggests applying ultrasound at a medium intensity ($0.3-1.2 \text{ W/cm}^2$) *in vivo* in order to damage a specific area of the donor organ such that only stem cells are activated
20 and the organ can be regenerated, forming a hybrid organ that can be more compatible with the recipient.

Patent document WO 2005/013799 A1 discloses a method based on applying ultrasound *in vivo* during reperfusion, i.e., blood supply enters the tissue, after an
25 ischemia time. Ultrasound is only applied for a short time span (not more than 15 minutes) to favor the entrance of blood and oxygen into a tissue and to reduce microcirculation disorders caused by reperfusion.

Therefore, it can be inferred from the art that applying ultrasound generates heat in
30 both aqueous media and tissues. Ultrasound applied for only between 1-10 minutes (times used in most medical applications) and at frequencies and intensities that are usual in the art alone causes the heating of tissues, which is interpreted as a negative factor for proper storage of an *ex vivo* biological sample. When the

temperature exceeds 38°C, exposure to ultrasound is usually interrupted because it damages tissues. For example, patent document US 5,267,985 discloses the possibility of using ultrasound for 15-30 minutes to achieve tissue penetration between 1 and 2 cm.

In fact, ultrasound has often been applied in clinical practice precisely as therapy to cause the heating of tissue. Besides the heating caused by ultrasound alone, ceramic transducers tend to heat up with vibration, even further increasing the thermal effect on tissue (Watson T et al., 2008, 48: 321-329; Baker KG, et al., 2001; 81: 1351-1358; Legay M et al.; 2011, ID670108; 1-17).

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Summary of the invention

It would be desirable to provide a device for transport and preservation of an ex vivo biological sample and a method of the type indicated above that reduces injury to the biological sample, and accordingly increases viability thereof in comparison to what could be obtained with known devices. The invention also considers, in the case of transplants, the problem of improving the quality of life of transplant patients, and in the worst cases, of significantly reducing the need for another transplant.

One aspect of the present invention provides a device for transport and preservation of an ex vivo biological sample for subsequent transplant in a living human being or animal, comprising a chamber for containing said biological sample, delimited by

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walls made of a thermal insulating material and cooling means for keeping the temperature inside said chamber below the temperature outside said device, wherein the device further comprises an auxiliary container containing a preservation solution, without external oxygen delivery and for containing said biological sample immersed in said preservation solution and at least an ultrasound system suitable for generating and applying ultrasound on said biological sample, and wherein said auxiliary container is closable.

Therefore, contrary to what was expected, it has been verified in the invention that applying ultrasound, which is applied such that it does not generate local heating in

the sample, does not negatively affect the positive effect of cooling the biological sample, and accordingly does not damage it either. Furthermore, however, it has surprisingly been verified that a very significant improvement is achieved with ultrasound, so much so that as will be seen later in the description, a synergistic effect between applying cold conditions inside the chamber of the device and applying ultrasound could be confirmed. To that end, there are many possible configurations, such as applying ultrasound close to the biological sample at a low intensity, or arranging the transducers away from the sample at higher intensities and in contact with a preservation fluid containing the sample.

Furthermore, also contrary to what was expected, problems with the duration of applying ultrasound were not verified in the invention. In other words, the need to keep the sample under cold ischemia conditions for several hours is known in organ transport. According to the invention, ultrasound can be continuously applied at suitable intensities, and despite what would be expected, it does not damage the biological sample either.

Therefore, by applying ultrasound, and preventing local heating of the sample, a significant reduction of cell damage has been verified. Furthermore, based on the assays conducted, it has been verified that protection of the organ against injury induced by cold ischemia by simultaneously applying both treatments is unexpectedly better than the sum of all the protection obtained through the treatments separately.

Furthermore, the invention covers a series of preferred features the usefulness of which will be highlighted below in the detailed description of an embodiment of the invention.

In a preferred embodiment, it has been verified that said ultrasound system is suitable for emitting said ultrasound at a frequency comprised between 25 kHz and 1 MHz and a sound intensity comprised between 0.01 and 2 W/cm², and preferably

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between 0.02 and 1 W/cm², and particularly preferably between 0.02 and 0.1 W/cm². These frequency and intensity ranges have been verified to be particularly beneficial in reducing cell damage. Particularly at a lower intensity, better results are obtained and less cooling of the sample and/or separation of the transducers with
5 respect to the biological sample is necessary, which allows the device to be more compact.

It can also preferably be envisaged that the ultrasound is applied in a continuous manner or alternatively in a pulsed manner. Furthermore, the invention does not rule
10 out simultaneously applying ultrasound having different frequencies by means of the corresponding individual control of each of the transducers of the device.

It has also been verified that the lower the temperature, the greater the synergistic effect obtained with the ultrasound. Therefore, in a particularly preferred manner, the
15 cooling means are suitable for keeping the temperature inside said chamber between 0 and 15°C, and preferably between 2 and 10°C, and particularly preferably between 2 and 6°C. So much so that as has been verified, the combination of cold conditions and ultrasound together provides better results than what would be expected in the best case of the sum of the effects of cold conditions
20 and ultrasound separately.

In a preferred embodiment, the device according to the invention comprises a sheet-like support in said chamber suitable for supporting the biological sample, said sheet-like support being able to vibrate freely when said ultrasound is applied, and
25 the ultrasound system comprises at least one transducer mounted on at least one of the walls of said sheet-like support. As a result, the damping effect of the support is minimized and ultrasound is introduced in the biological sample in a more efficient manner, improving the effect thereof in bulkier biological samples provided that excessive local heating in the biological sample is prevented.

30

In a particularly preferred manner, said at least one transducer is mounted on the face of said sheet-like support opposite the support surface for said biological sample to apply said ultrasound towards said support surface. A more homogenous

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ultrasonic field is thereby obtained, even further favoring the combined effect of cold conditions and ultrasound on the biological sample. It may be appropriate for the sheet-like support to be a tray and for such tray to allow containing a fluid, such as water, to improve transmission of the applied ultrasound. The function of the fluid is
5 on one hand to act as a coolant, and to simultaneously favor transmitting ultrasound to the biological sample.

In a particularly preferred manner, said sheet-like support is made of metal in order to increase the rigidity thereof and prevent damping to the greatest extent, and to
10 achieve more direct transmission of the ultrasound on the biological sample.

In a particularly preferred embodiment, the device comprises a closable auxiliary container containing a preservation solution. Again, it has been verified that the combination of cold conditions and ultrasound plus immersion of the biological
15 sample in a preservation solution provides better results than the sum of their individual effects as regards reducing damage to the biological sample.

In an embodiment of the device according to the invention, the sheet-like support is a tray adapted for containing a fluid.
20

In another preferred embodiment, the sheet like support is a tray containing water.

In another embodiment the auxiliary container comprises a false bottom located away from the base of said auxiliary container, and said false bottom being in fluid
25 communication with the rest of said auxiliary container.

Also in a particularly preferred manner, the auxiliary container can incorporate a false bottom intended for keeping the biological sample away from the support surface of the container, said false bottom being in fluid communication with the rest
30 of the auxiliary container. As a result, the sample can be placed in the auxiliary container as if it were floating in the preservation solution. The sample therefore does not receive such a direct action of the ultrasound and can work at higher intensities.

Furthermore, a second aspect of the present invention provides a method for transport and preservation of an *ex vivo* transplantable biological sample comprising comprises the steps of removing and washing blood out of the biological sample, placing the biological sample in a chamber delimited by walls made of a thermal insulating material without external oxygen delivery and irradiating the sample with ultrasound. More particularly, in the method according to the invention the biological sample is kept immersed in a preservation solution without external oxygen delivery by placing said biological in a chamber delimited by walls made of a thermal insulating material.

Preferably, said biological sample is placed immersed in an auxiliary container, which is closable, containing said preservation solution, and said auxiliary container is placed in said chamber.

In one embodiment of the method, in the irradiation step for irradiating said biological sample the ultrasound has frequencies comprised between 25 kHz and 1 MHz and a sound intensity comprised between 0.01 and 2 W/cm², and particularly preferably between 0.02 and 1 W/cm², and particularly preferably between 0.02 and 0.1 W/cm².

In another embodiment, the method comprises a cooling step for cooling the temperature in the chamber to a temperature between 0 and 15°C, and more preferably between 2 and 10°C, and in a particularly preferred manner from 2 to 6°C.

In another embodiment, the method further comprises a step consisting of keeping said biological sample immersed in a preservation solution.

Finally the method further comprises a step of placing said auxiliary container on a sheet-like support that is a tray adapted for containing a fluid and said tray being able to vibrate freely when said ultrasound is applied.

- 10 -

The invention also refers to the use of a device for transport and preservation of an *ex vivo* biological sample. With the device, the biological sample is maintained immersed in a preservation solution without oxygen delivery under hypothermal conditions and said sample is irradiated with ultrasound such that the viability,
5 functionality and interaction between the different cells of said biological sample are preserved for said biological sample to be used in subsequent laboratory research.

Furthermore, the invention also covers other detail features illustrated in the detailed description of an embodiment of the invention and in the attached drawings.

10

Brief description of the drawings

Further advantages and features of the invention will become apparent from the following description, in which, without any limiting character, preferred
15 embodiments of the invention are disclosed, with reference to the accompanying drawings in which:

Figure 1 shows an exploded perspective view of a first embodiment of a device for transport of an *ex vivo* biological sample.

20 Figure 2 shows a longitudinally sectioned front view of the device of Figure 4.

Figure 3 shows a top plan view of a first embodiment of the support tray for the biological sample of the device of Figure 1.

Figure 4 shows a side view of the tray of Figure 3.

Figure 5 shows a second embodiment of the support tray for the biological sample.

25 Figure 6 shows a second embodiment of the device for transport according to the invention.

Figure 7A shows a third embodiment of the device for transport according to the invention.

30 Figure 7B shows an alternative embodiment of the auxiliary container of the device of Figure 7A.

Figures 8A to 13 show percentage of protection of biological samples of liver and kidney with respect to conditions in the state of the art.

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Detailed description of embodiments of the invention

Figures 1 and 2 show a first embodiment of the device 1 for transport and preservation of an *ex vivo* transplantable biological sample 100 according to the invention. In a particularly preferred manner, the device according to the invention is a portable cooler.

As has already been previously indicated, the biological sample 100 comprises both organs that can be transplanted between donor and recipient, which can be human or animal, and tissues. Furthermore, the biological sample can also be intended for research without necessarily having to be transplanted.

The device 1 for transport and preservation according to the invention has a parallelepiped-shaped main isothermal container 16 open on the upper face. The walls 4 made of a thermal insulating material of the main container 16, including its upper lid, delimitate a chamber 2 intended for containing the biological sample 100.

Cooling means 6 suitable for keeping the temperature inside the chamber 2 below the temperature outside said device 1 are provided inside the main container 16. These cooling means 6 can comprise solutions as different as ice blocks, cooling gel packs or more complex solutions comprising a compressor and a heat exchanger. The device 1 could also consist of a transportable cooler with or without an external power supply. Nevertheless, it is desirable for the solution to be as light as possible in order to not affect transportability of the assembly. The cooling means 6 allow keeping the temperature inside the chamber 2 between 0 and 15°C. In another preferred embodiment, the temperature inside the chamber 2 is kept between 2 and 10°C, and particularly preferably between 2 and 6°C.

The device 1 comprises in the central part a sheet-like support 10 by way of a metal tray supported inside the chamber 2 such that it can vibrate freely. The tray of this first embodiment can be seen in detail in Figures 3 and 4. An aluminum tray with less than 1 mm, and preferably 0.5 mm thick, is used in the embodiment.

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Furthermore, the device comprises an ultrasound system suitable for generating and applying ultrasound on the biological sample 100. To that end, the ultrasound system according to the drawings has an electric signal generator 20, an amplifier 22, a battery 24, and in this case four piezoelectric transducers 8.

5

Vibrations are applied to the sheet-like support 10 by way of an aluminum tray through the four transducers 8. Said transducers 8 are mounted on the lower face of the tray. As a result of this configuration, transmission between the transducers and the biological sample 100 is more direct, because vibrations are generated directly
10 below the lower part of the biological sample. In a particularly preferred manner, as can be seen in Figure 6, the tray can additionally contain water, but to improve transmission of ultrasound, it is envisaged that the biological sample 100 is contained in a bag or container filled with preservation solution. The preservation solutions contemplated herein are, for example, Lactated Ringer's solution, Celsior
15 solution or University of Wisconsin solution.

In an alternative embodiment shown in Figure 5, the tray can have transducers 8 on the side walls to generate a lateral vibrational field. The transducers 8 could also alternatively be located both on side walls and on the bottom of the tray.

20

The ultrasound transducers 8 transmit mechanical waves having a frequency comprised between 25 kHz and 1 MHz and a sound intensity comprised between 0.1 and 2 W/cm² into said chamber 2 during transport of said biological sample 100. The intensity can preferably be comprised between 0.02 and 1 W/cm², and still more
25 preferably between 0.02 and 0.1 W/cm². The higher the sound intensity used, the further away the transducers 8 will be and/or the larger the amount of preservation fluid or coolant will be placed in the device 1 to meet the objective of preventing local heating in the biological sample 100. Furthermore, according to the invention the ultrasound can be applied continuously. Alternatively, the ultrasound can also be
30 applied intermittently, i.e., it is not applied during the entire time transport lasts. Alternatively, it can also be applied in a pulsed manner and/or at different frequencies, either continuously or intermittently.

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In a particularly preferred manner, the biological sample 100 is contained in a receptacle containing a preservation solution, which improves the results of applying ultrasound.

- 5 Other embodiments of the device 1 for transport according to the invention which share many of the same features described in the preceding paragraphs are shown below. Accordingly, only those elements that are different between such embodiments will be described hereinafter, whereas for common elements reference is made to the description of the first embodiment.

10

The embodiment of the device 1 of Figure 6 differs primarily in that the sheet-like support 10 is integrated directly in the walls 4 of the main container 16.

- 15 Finally, in the embodiment of the device in Figure 7A, an auxiliary container 14 is provided inside the main container 16 with a preservation solution. In a particularly preferred manner, the auxiliary container 14 is closable, with rigid walls and a lid hinged on one of its walls. In a particularly preferred manner, when the transducers are located in the lower part of the tray, the auxiliary container 14 has dimensions intended for occupying the entire emission surface of the transducers 8. As a result
- 20 of the auxiliary container 14 being filled with preservation solution, the ultrasound reaches the biological sample 100 in a more efficient manner. In a particularly preferred manner, the auxiliary container 14 is manufactured by thermoforming using a formable plastic, such as for example high density polyethylene, polypropylene, polyethylene terephthalate or the like. Also in a particularly preferred
- 25 manner, the material will be transparent to enable seeing the biological sample 100 without needing to open the auxiliary container 14.

- Figure 7A shows an alternative embodiment of the auxiliary container 14. In this case, the auxiliary container 14 comprises a false bottom 18 located away from the
- 30 base of said auxiliary container 14, and said false bottom 18 being in fluid communication with the rest of said auxiliary container 14. The false bottom thereby places the biological sample 100 away from the support surface of the auxiliary container 14. In this case, the false bottom 18 consists of a sheet of rigid plastic

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supported on the side walls of the auxiliary container 14. Then to achieve proper fluid communication, a plurality of openings 20 that allow the passage of the preservation solution is provided in this case, such that said solution receives the direct effect of the ultrasound coming from the lower transducers 8.

5

Finally, in a particularly preferred manner, the auxiliary container 14 contains a preservation fluid under sterile conditions from the group consisting of Lactated Ringer's preservation solution, Celsior preservation solution or University of Wisconsin preservation solution. This allows a much more hygienic handling of the biological sample under more suitable conditions for the proper preservation thereof.

The embodiments described up until now represent non-limiting examples, such that the person skilled in the art will understand that beyond the examples that are shown, a number of combinations of the claimed features are possible within the scope of the invention.

Experimental examples

Different tests conducted to put the method according to the invention into practice are described below.

Methodology

The experimental protocol was carried out from livers and kidneys of Landrace pigs. The kidney and liver were perfused with preservation solution at 4°C to remove blood contained in the organ, and the organ was kept under cold conditions, bathed in ice until the organs were immersed in different preservation solutions. Next the biological samples were placed in the cooler and kept in the cooler with or without ultrasound for 8 hours (liver) and 24 hours (kidney). All the procedures were performed under anesthesia and the study respected the European Union regulations concerning experiments using animals (Directive 86/609/EEC).

Experimental design

Protocol 1

The experimental groups formed were the following:

- 5 **GROUP 1:** cold ischemia group in the conventional transport system and with preservation solution. This group was divided into different subgroups depending on the preservation solution used.
- 10 1.1) Perfusion of liver grafts (n=10) and kidney grafts (n=10) in Lactated Ringer's solution at 4°C and preservation of such organs with Lactated Ringer's solution in the conventional transport system for 8 and 24 hours for liver and kidney, respectively, between 2-6°C;
- 1.2). The same as 1.1 but using Celsior solution for washing and preserving the organ;
- 15 1.3) The same as 1.1 but using UW (University of Wisconsin) solution for washing and preserving the organ.
- GROUP 2:** cold ischemia group in the system with ultrasound (25 kHz and 0.04 W/cm²) and without preservation solution: Perfusion of liver grafts (n=10) and kidney grafts (n=10) in Lactated Ringer's solution at
- 20 4°C to wash the organs for the purpose of removing blood contained therein. After washing, the organs were stored for 8 and 24 hours for liver and kidney, respectively, and without preservation solution in the transport system with ultrasound (25 kHz and 0.04 W/cm²) between 2-6°C.
- 25 **GROUP 3:** cold ischemia group in the transport system with ultrasound (25 kHz and 0.04 W/cm²) and with preservation solution. The group was divided into different subgroups depending on the preservation solution used.
- 30 3.1) Perfusion of liver grafts (n=10) and kidney grafts (n=10) in Lactated Ringer's solution at 4°C. The organs were then stored with Lactated Ringer's solution for 8 and 24 hours for liver and kidney,

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respectively, in the transport system with ultrasound at a temperature comprised between 2-6°C;

3.2). The same as 3.1 but using Celsior solution for washing and preserving the organ;

5 3.3) The same as 3.1 but using UW solution for washing and preserving the organ.

Experiments were also performed to evaluate if other frequencies and/or intensities could provide more protection than what was achieved with frequencies of 25 kHz
10 and an intensity of 0.04 W/cm². To do this, the following experiments were performed.

GROUP 4: cold ischemia group in the system with ultrasound (40, 80, 200, 580 kHz and 1 MHz) and intensity of 0.04 W/cm² and with
15 preservation solution: The group was divided into different subgroups depending on the frequency used.

4.1) Perfusion of liver grafts (n=10) and kidney grafts (n=10) in UW solution at 4°C. The organs were then stored in UW solution for 8 and 24 hours for liver and kidney, respectively, in the transport system
20 with ultrasound at 40 kHz and intensity of 0.04 W/cm² and between 2-6°C;

4.2). The same as 4.1 but using 80 kHz and intensity of 0.04 W/cm² and between 2-6°C;

4.3) The same as 4.1 but using 200 kHz and intensity of 0.04 W/cm² and between 2-6°C;
25

4.4) The same as 4.1 but using 580 kHz and intensity of 0.04 W/cm² and between 2-6°C;

4.5) The same as 4.1 but using 1 MHz and intensity of 0.04 W/cm² and between 2-6°C.

30

Experiments were also performed to verify the effect of ultrasound at an intensity greater than 0.04. The following experiments were performed for that purpose:

GROUP 5: cold ischemia group in the system with ultrasound (25 kHz and intensity of 0.1 W/cm^2 and with preservation solution). Perfusion of liver grafts (n=10) and kidney grafts (n=10) in UW solution at 4°C . The organs were then stored for 8 and 24 hours for liver and kidney, respectively, in the transport system with ultrasound at 25 kHz and intensity of 0.1 W/cm^2 and at a temperature comprised between $2-6^\circ\text{C}$.

The following experimental groups were further added to evaluate the effect of the preservation solution and the cold conditions:

10

GROUP 6: cold ischemia group in the conventional transport system but without preservation solution. Perfusion of liver grafts (n=10) and kidney grafts (n=10) with Lactated Ringer's solution at 4°C to wash the organs for the purpose of removing blood contained in the organs. After washing, the organs were stored without preservation solution in the conventional cooler (without ultrasound) for 8 and 24 hours for liver and kidney, respectively, between $2-6^\circ\text{C}$.

GROUP 7: non-cold condition ischemia group and combined with ultrasound (25 kHz and intensity of 0.04 W/cm^2) or not. The group was divided into two subgroups.

7.1) Perfusion of liver grafts (n=10) and kidney grafts (n=10) in UW solution at a temperature between $20-25^\circ\text{C}$. The organs were then stored for 8 and 24 hours for liver and kidney, respectively, at a temperature comprised between $20-25^\circ\text{C}$;

7.2) Perfusion of liver grafts (n=10) and kidney grafts (n=10) in UW solution at a temperature comprised between $20-25^\circ\text{C}$. The organs were then stored for 8 and 24 hours for liver and kidney, respectively, at a temperature comprised between $20-25^\circ\text{C}$ and with ultrasound at 25 kHz and intensity of 0.04 W/cm^2 .

Collecting and processing samples

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At the end of ischemia, and in all the experimental groups and subgroups, liver and kidney perfusate samples were collected to assess the liver and kidney damage induced by ischemia using widely standardized techniques. Liver damage was evaluated by determining transaminase levels in the perfusate and by means of
5 determining the caspase 3 activity in liver tissue. Kidney damage was evaluated by means of determining lactate dehydrogenase in the perfusate and caspase 3 activity in kidney tissue. MDA (malondialdehyde) levels were determined in liver and kidney tissue samples as an oxidative stress index, and ATP (adenosine triphosphate)
10 levels were determined as an organ energy metabolism preservation index (Salahudeen AK *et al.*, Am J Transpl 2003; 3:273-280; Omar R *et al.*, Gut 1989;30:510-514; Peralta *et al.*, Am J Physiol.2000; 279:G163-71).

The statistical study was conducted by means of an analysis of variance (ANOVA),
15 and the level of statistical significance was then determined with a Student-Newman-Kels test.

The results obtained and shown schematically in the drawings are explained below in detail.

20

Figures 8A to 8E: show the percentage of protection, or in other words, the percentage of reduction of liver injury in biological samples consisting of liver grafts under conditions 1 to 6 described below versus injury induced by the following condition: UW solution + cold conditions (2-6°C) when the parameters indicative of
25 liver damage, namely, AST (aspartate aminotransferase), ALT (alanine aminotransferase), caspase 3 activity, MDA (malondialdehyde) and ATP (adenosine triphosphate), were evaluated at the end of the 8 hours of cold ischemia.

Conditions (1-6):

- 1: UW solution, ultrasound frequency of 25 kHz and cold conditions (2-6°C).
- 30 2: UW solution, ultrasound frequency of 40 kHz and cold conditions (2-6°C).
- 3: UW solution, ultrasound frequency of 80 kHz and cold conditions (2-6°C).
- 4: UW solution, ultrasound frequency of 200 kHz and cold conditions (2-6°C).
- 5: UW solution, ultrasound frequency of 580 kHz and cold conditions (2-6°C).

6: UW solution, ultrasound frequency of 1 MHz and cold conditions (2-6°C).

An ultrasound intensity of 0.04 W/cm² was applied in all of cases 1 to 6.

Figures 9A to 9D: show the percentage of protection in biological samples consisting of kidney grafts in conditions 1 to 6 described below versus injury induced by the following condition: UW solution + cold conditions (2-6°C) when the parameters indicative of kidney damage, namely, LDH (lactate dehydrogenase), caspase 3 activity, MDA and ATP, were evaluated at the end of the 24 hours of cold ischemia.

- 10 1: UW solution, ultrasound frequency of 25 kHz and cold conditions (2-6°C).
- 2: UW solution, ultrasound frequency of 40 kHz and cold conditions (2-6°C).
- 3: UW solution, ultrasound frequency of 80 kHz and cold conditions (2-6°C).
- 4: UW solution, ultrasound frequency of 200 kHz and cold conditions (2-6°C).
- 5: UW solution, ultrasound frequency of 580 kHz and cold conditions (2-6°C).
- 15 6: UW solution, ultrasound frequency of 1 MHz and cold conditions (2-6°C).

An ultrasound intensity of 0.04 W/cm² was applied in all of cases 1 to 6.

Figure 10: shows the percentage of protection in liver versus injury induced by the following condition: non use of preservation solution + cold conditions (2-6°C) + no ultrasound when the parameter indicative of liver damage, AST, was evaluated at the end of the 8 hours of cold ischemia.

- 1: Lactated Ringer's solution, without ultrasound and with cold conditions (2-6°C).
- 2: Celsior solution, without ultrasound and with cold conditions (2-6°C).
- 25 3: UW solution, without ultrasound and with cold conditions (2-6°C).
- 4: Lactated Ringer's solution, with ultrasound (25 KHz, 0.04 W/cm²) and with cold conditions (2-6°C).
- 5: Celsior solution, with ultrasound (25 KHz, 0.04 W/cm²) and with cold conditions (2-6°C).
- 30 6: UW solution, with ultrasound (25 KHz, 0.04 W/cm²) and with cold conditions (2-6°C).

- 20 -

Note: out of the preservation solutions used, the standard solution in clinical practice is UW (University of Wisconsin) preservation solution. Lactated Ringer's solution does not contain drugs and contains only mineral salts. Celsior solution contains glutathione, and UW solution contains more drugs, such as adenosine, glutathione
 5 and allopurinol.

Figure 11: shows the percentage of protection in kidney versus injury induced by the following condition: non use of preservation solution + cold conditions (2-6°C) + no ultrasound when the parameter indicative of kidney damage, LDH, was evaluated
 10 at the end of the 24 hours of cold ischemia.

- 1: Lactated Ringer's solution, without ultrasound and with cold conditions (2-6°C).
- 2: Celsior solution, without ultrasound and with cold conditions (2-6°C).
- 3: UW solution, without ultrasound and with cold conditions (2-6°C).
- 15 4: Lactated Ringer's solution, with ultrasound (25 KHz, 0.04 W/cm²) and with cold conditions (2-6°C).
- 5: Celsior solution, with ultrasound (25 KHz, 0.04 W/cm²) and with cold conditions (2-6°C).
- 6: UW solution, with ultrasound (25 KHz, 0.04 W/cm²) and with cold
 20 conditions (2-6°C).

Figure 12: shows the percentage of protection in liver versus injury induced by the following condition: UW preservation solution + without cold conditions (20-25°C) + no ultrasound when the parameter indicative of liver damage, AST, is evaluated at
 25 the end of the 8 hours of cold ischemia.

- 1: UW solution without ultrasound and with cold conditions (2-6°C).
- 2: UW solution with ultrasound and without cold conditions (20-25°C).
- 3: Without preservation solution, with ultrasound and with cold conditions (2-6°C).
- 30 4: UW solution, with ultrasound (25 kHz, 0.04 W/cm²) and with cold conditions (2-6°C). Expected result: Protection 1 + Protection 2= Protection (1+2)

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5: UW solution, with ultrasound (25 kHz, 0.04 W/cm²) and with cold conditions (2-6°C). Result actually observed. Protection 1 + Protection 2 < Protection (1+2).

5 **Figure 13:** shows the percentage of protection in kidney versus injury induced by the following condition: UW preservation solution + without cold conditions (20-25°C) + no ultrasound when the parameter indicative of kidney damage, LDH, is evaluated at the end of the 24 hours of cold ischemia.

1: UW solution without ultrasound and with cold conditions (2-6°C).

10 2: UW solution with ultrasound and without cold conditions (20-25°C).

3: Without preservation solution, with ultrasound and with cold conditions (2-6°C).

4: UW solution, with ultrasound (25 kHz, 0.04 W/cm²) and with cold conditions (2-6°C). Expected result: Protection 1+ Protection 2 = Protection (1+2).

15 5: UW solution, with ultrasound (25 kHz, 0.04 W/cm²) and with cold conditions (2-6°C). Result actually observed. Protection 1+ Protection 2 < Protection (1+2).

20 Discussion of the experimental results

It is important to point out that as was expected, without applying ultrasound the temperature ranged between 2-6°C inside the cooler, in the preservation fluid and in the organ. In addition, applying ultrasound under cold conditions (2-6°C) did not
25 modify the temperature inside the cooler, which was between 2-6°C for all the experiments, but organ and preservation solution cooling was lost.

When applying ultrasound, the temperature ranged between 2-6°C inside the cooler, the temperature of the preservation fluid ranged between 14-16°C and the
30 temperature of the organ between 16-18°C.

The effect of heating caused by the ultrasound is an expected result according to background documents of interest. Nevertheless, contrary to what would be

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expected, taking into account the vital importance of cooling the organ (4°C) and keeping the temperature of the preservation fluid and the organ between 2-6°C to store organs under hypothermal ischemia conditions, the results indicate that applying ultrasound does not negatively affect the effect of cooling the biological
5 sample, and accordingly does not damage it.

Furthermore, however, it has surprisingly been verified that a very significant improvement is achieved, so much so that a synergistic effect between applying cold conditions inside the chamber of the device and applying ultrasound could be
10 confirmed.

Figures 8A to 8E: as shown in these drawings, under all conditions (at different frequencies and the same intensity of 0.04 W/cm²), applying ultrasound protects the liver graft under cold ischemia conditions, said protective effects being more evident
15 under condition 1, i.e., at a frequency of 25 kHz. Other results not shown in the drawings indicate that at higher intensities, as is the case of 0.1 W/cm², protection of the liver graft is also obtained. For example, a percentage of protection or a reduction of injury of 55% is obtained at frequencies of 25 kHz and an intensity of 0.1 W/cm² versus the condition: UW solution + cold conditions (2-6°C), when the
20 parameter for liver damage, AST, is evaluated at the end of the 8 hours of cold ischemia.

Figures 9A to 9D: show the same pattern of kidney protection as that shown in Figures 8A to 8E for the liver.

25

Figure 10: as was expected, protection of the liver provided by the preservation solutions without applying ultrasound (conditions 1 to 3) is observed; protection of the liver graft is better if the UW preservation solution is used with respect to both solutions (Ringer or Celsior), and protection obtained by the Celsior solution is better
30 than that obtained by Lactated Ringer's solution. In addition, when observing protection provided by the preservation solutions in the presence of ultrasound (conditions 4-6), unexpected results are obtained, indicating better protection of the ultrasound, but such protection is similar under all conditions (4-6). In other words,

the same degree of protection is obtained regardless of the preservation solution used, whether it is Lactated Ringer's solution, Celsior solution or UW solution.

Figure 11: shows the same pattern of kidney protection as that shown in Figure 10
5 for the liver.

Figure 12: as was expected, cold conditions without applying ultrasound (condition 1) and using the UW solution increases protection of the liver graft by about 30% when compared with preservation with UW at 4°C and at room temperature (20-
10 25°C). Unexpectedly, the protection obtained by condition 2 (UW solution, in the presence of ultrasound and at room temperature) is better than that of condition 1 (UW solution and cold without ultrasound). In other words, in the presence of preservation solution at 4°C, it is better to apply ultrasound in a chamber at room temperature than under hypothermal conditions (chamber at a temperature between
15 2-6°C) and without ultrasound. The results indicating that protection obtained under condition 3 (without preservation solution, under cold conditions and with ultrasound) is better than that obtained under conditions 1 and 2 are also unexpected. These results indicate that it is better to combine ultrasound and cold conditions (even without the presence of a preservation solution) than to combine
20 the presence of a preservation solution with cold conditions (condition 1) or with ultrasound (condition 2). Conditions 4 and 5 show the expected and observed protection, respectively, when using UW solution, ultrasound and cold conditions. The expected protection would be, in the best case, the sum of protection obtained in condition 1 and of protection obtained in condition 2. In other words, due to the
25 heat effect induced by ultrasound, protection 1 + 2 would not be expected to correspond to the sum of conditions 1 and 2 considered separately. However, these were not the results observed. The obtained results indicated a synergistic effect when both treatments (cold conditions and ultrasound) are combined because the protection obtained when both treatments are combined is much better than the sum
30 of protections obtained when both treatments are applied separately. Furthermore, the protection obtained when both treatments are combined and in the presence of preservation solution is much better than that obtained when both treatments are combined without preservation solution (condition 3).

Figure 13: shows the same pattern of kidney protection as that shown in Figure 5 for the liver.

- 5 Accordingly, taking all the results into account, the combination of preservation solution, ultrasound and cold conditions can be an extremely efficient strategy for transport and preservation of organs during cold ischemia.

10 A method and equipment for transporting and storing biological samples under hypothermal conditions and without oxygen delivery under better conditions than those currently available, and with effective transmission of ultrasound to the organ or tissue inside the chamber, are provided. All this allows reducing the harmful effects of cold ischemia and increasing viability of grafts before they are implanted in the recipient, thereby preventing having to do another transplant.

15

The equipment and method for preservation could also be useful in secondary organs; the number of organs available for transplant could accordingly increase, thereby reducing waiting lists. Furthermore, since the injuries induced by cold ischemia during the conservation and transport of organs is reduced, the time during which organs are transported in the cooler until they are implanted in the recipient can be extended. Furthermore, the equipment is easy to transport in order to prevent, among other factors, logistic complications resulting from dynamic preservation of the organ.

25

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A device for transport and preservation of an ex vivo biological sample for subsequent transplant in a living human being or animal, comprising a chamber for containing said biological sample, delimited by walls made of a thermal insulating material and cooling means for keeping the temperature inside said chamber below the temperature outside said device, wherein the device further comprises an auxiliary container containing a preservation solution, without external oxygen delivery and for containing said biological sample immersed in said preservation solution and at least an ultrasound system suitable for generating and applying ultrasound on said biological sample and wherein said auxiliary container is closable.

2. The device according to claim 1, wherein said ultrasound system is suitable for emitting said ultrasound at a frequency comprised between 25 kHz and 1 MHz and a sound intensity comprised between 0.01 and 2 W/cm².

3. The device according to claim 2, wherein said sound intensity is comprised between 0.02 and 1 W/cm², and preferably between 0.02 and 0.1 W/cm².

4. The device according to any one of claims 1 to 3, wherein said cooling means are suitable for keeping the temperature inside said chamber between 0 and 15°C.

5. The device according to claim 4, wherein said cooling means are suitable for keeping the temperature inside said chamber between 2 and 10°C, and preferably between 2 and 6°C.

6. The device according to any one of claims 1 to 5, comprising a sheet-like support in said chamber suitable for supporting said closable auxiliary container containing said biological sample, said sheet-like support being able to vibrate freely when said ultrasound is applied, and in that said ultrasound system comprises at least one transducer mounted on at least one of the walls of said sheet-like support.

7. The device according to claim 6, wherein said at least one transducer is mounted on the face of said sheet-like support opposite the support surface for said biological sample to apply said ultrasound towards said support surface.

5 8. The device according to claim 6 or 7, wherein said sheet-like support is made of metal.

9. The device according to any one of claims 6 to 8, wherein sheet-like support is a tray adapted for containing a fluid.

10 10. The device according to claim 9, wherein said sheet-like support is a tray adapted for containing water.

11. The device according to any one of claims 1 to 10, wherein said auxiliary
15 container comprises a false bottom located away from the base of said auxiliary container, and said false bottom being in fluid communication with the rest of said auxiliary container.

12. A method for transport of an ex vivo biological sample for subsequent
20 transplant in a living human being or animal, wherein the method comprises the following steps:

[a] removing blood from said biological sample under cold conditions and rapidly cooling said biological sample,

[b] keeping said biological sample immersed in a preservation solution without
25 external oxygen delivery by placing said biological sample in a chamber delimited by walls made of a thermal insulating material, and

[c] irradiating said sample with ultrasound.

13. The method according to claim 12, wherein said biological sample is placed
30 immersed in an auxiliary container, which is closable, containing said preservation solution, and said auxiliary container is placed in said chamber.

14. The method according to claim 12 or 13, wherein in said irradiation step for irradiating said biological sample, the ultrasound has frequencies comprised between 25 kHz and 1 MHz and a sound intensity comprised between 0.01 and 2 W/cm²

5 15. The method according to claim 14, wherein said ultrasound has an intensity comprised between 0.02 and 1 W/cm², and preferably between 0.02 and 0.1 W/cm².

10 16. The method according to any one of claims 12 to 15, further comprising a cooling step for cooling the temperature in said chamber to a temperature between 0 and 15°C.

15 17. The method according to claim 16, wherein in said cooling step the temperature in said chamber is kept between 2 and 10°C, and particularly preferably between 2 and 6°C.

20 18. The method according to any one of claims 12 to 17, further comprising a step of placing said auxiliary container on a sheet-like support that is a tray adapted for containing a fluid and said tray being able to vibrate freely when said ultrasound is applied.

25 19. Use of a device for transport and preservation of an ex vivo biological sample according to any one of claims 1 to 11, wherein said biological sample is maintained immersed in a preservation solution without oxygen delivery under hypothermal conditions and said sample is irradiated with ultrasound such that the viability, functionality and interaction between the different cells of said biological sample are preserved for said biological sample to be used in subsequent laboratory research.

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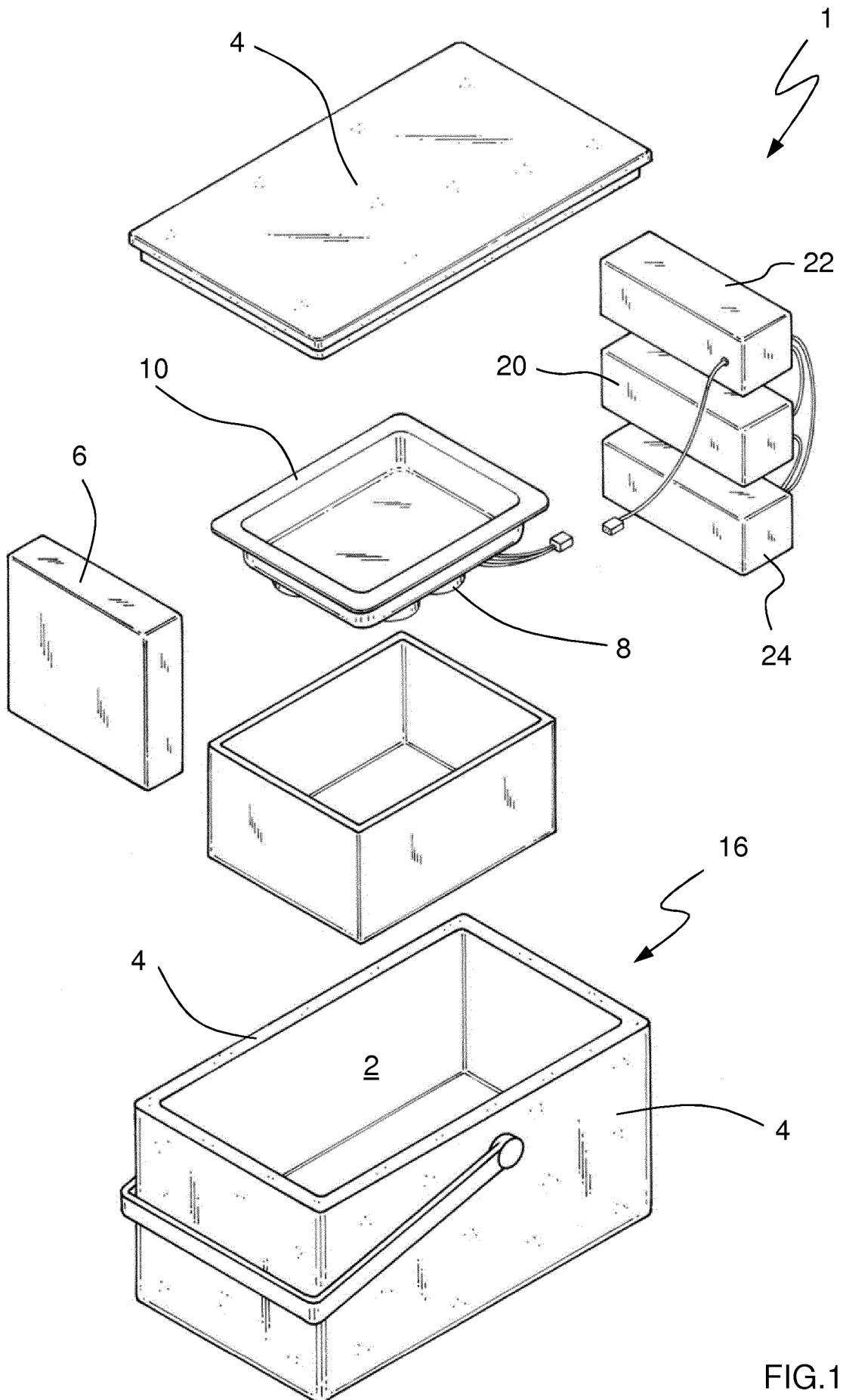
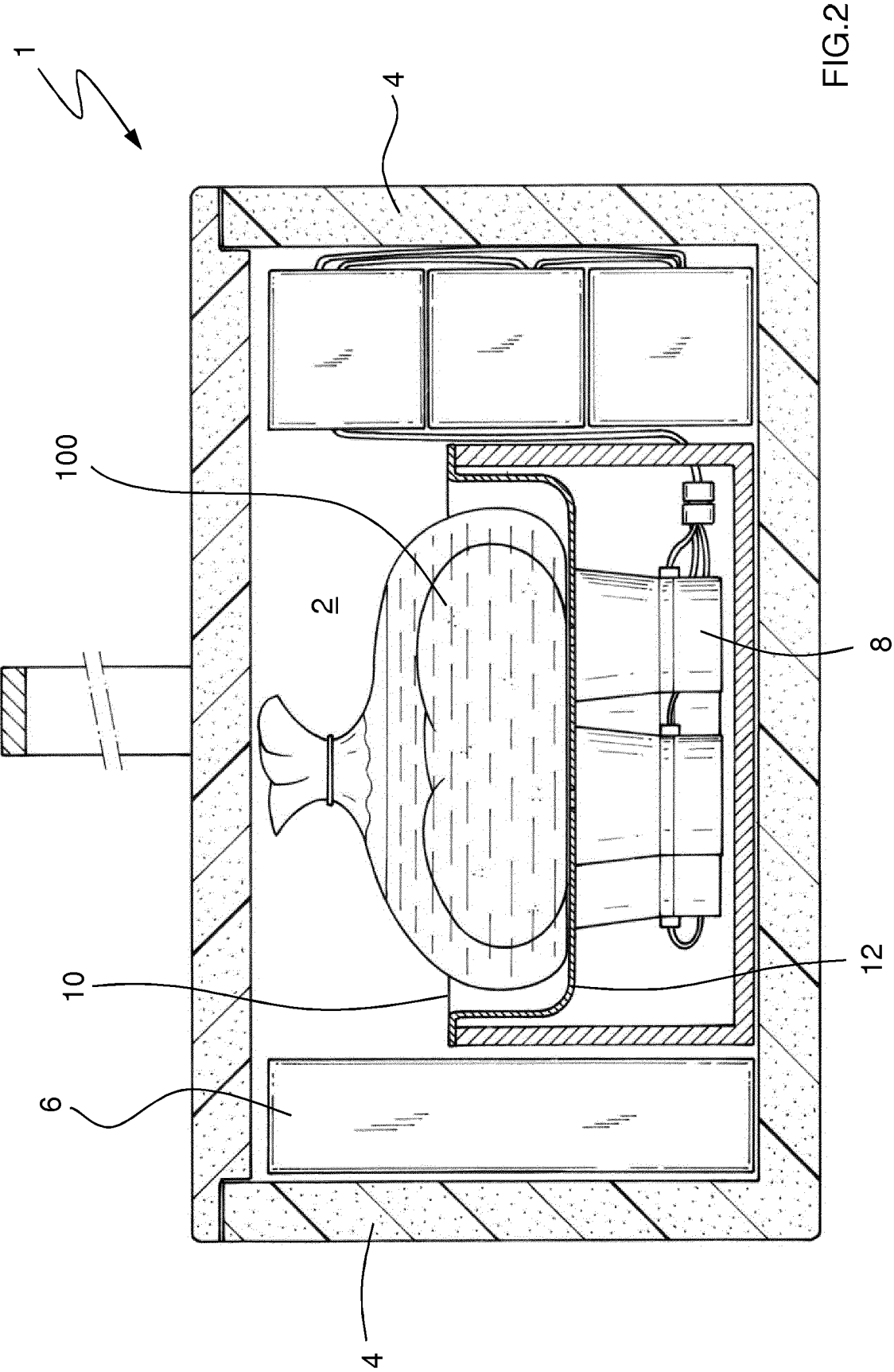


FIG.1



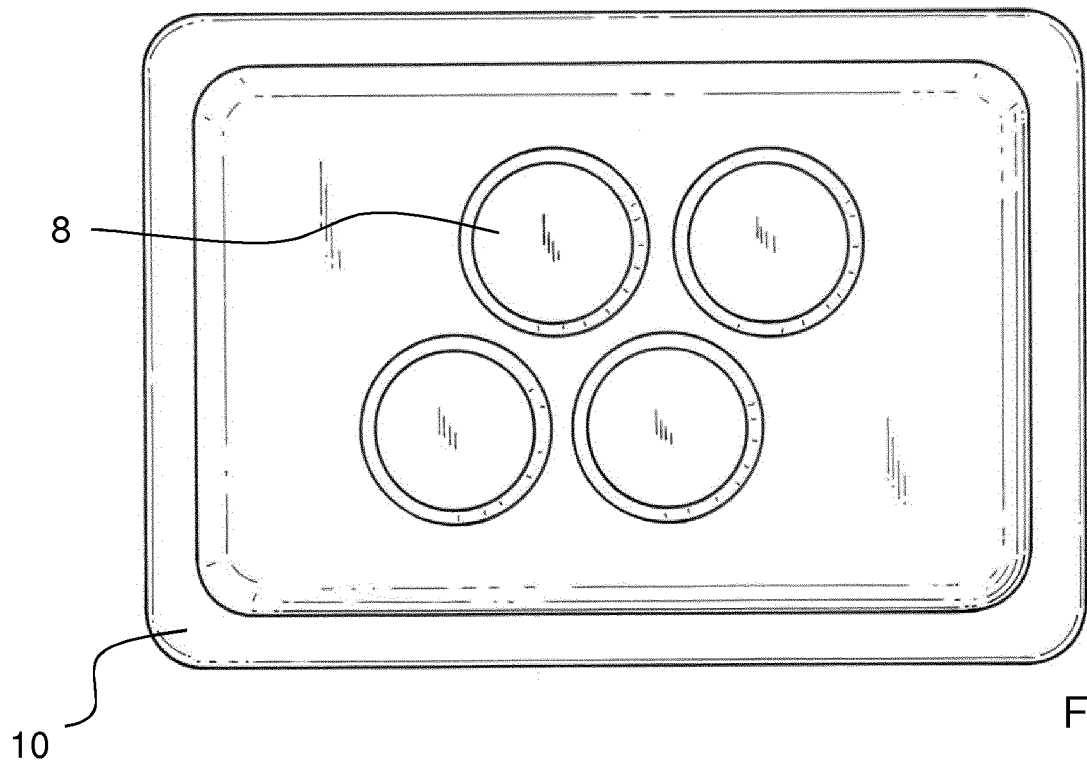


FIG.3

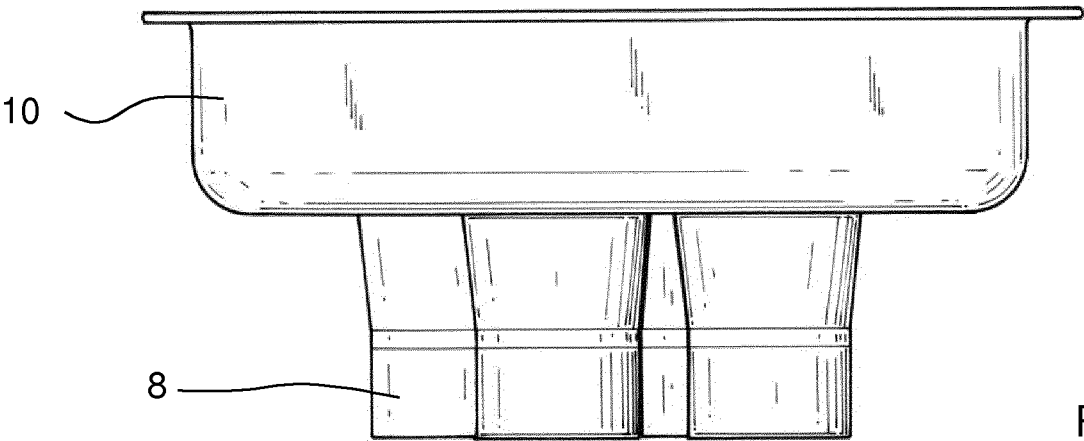


FIG.4

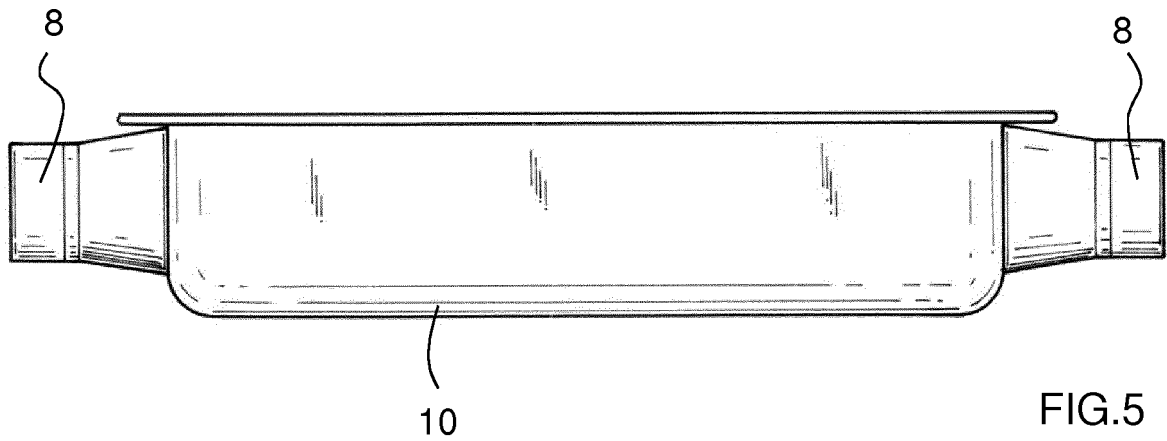


FIG.5

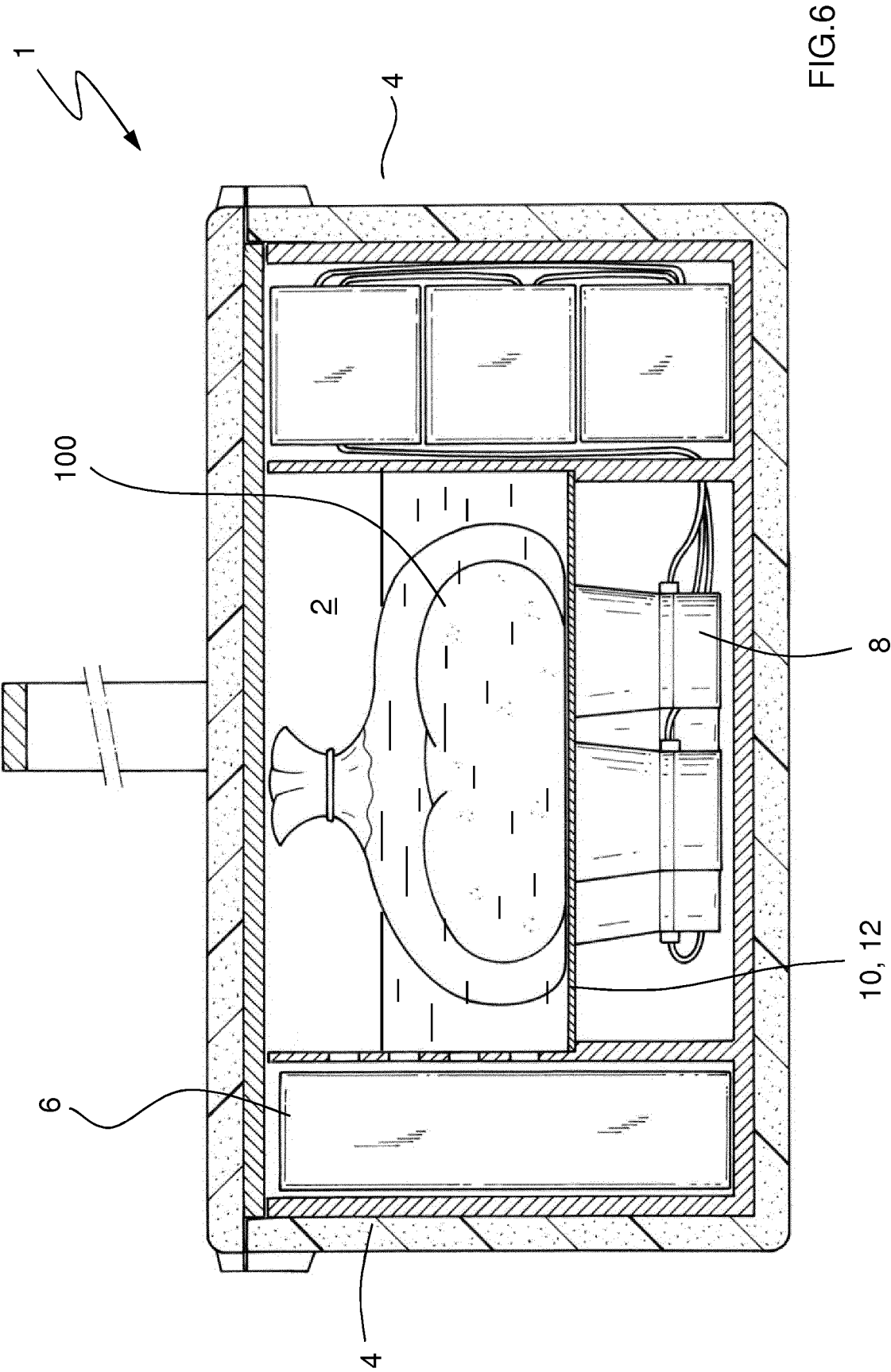


FIG. 6

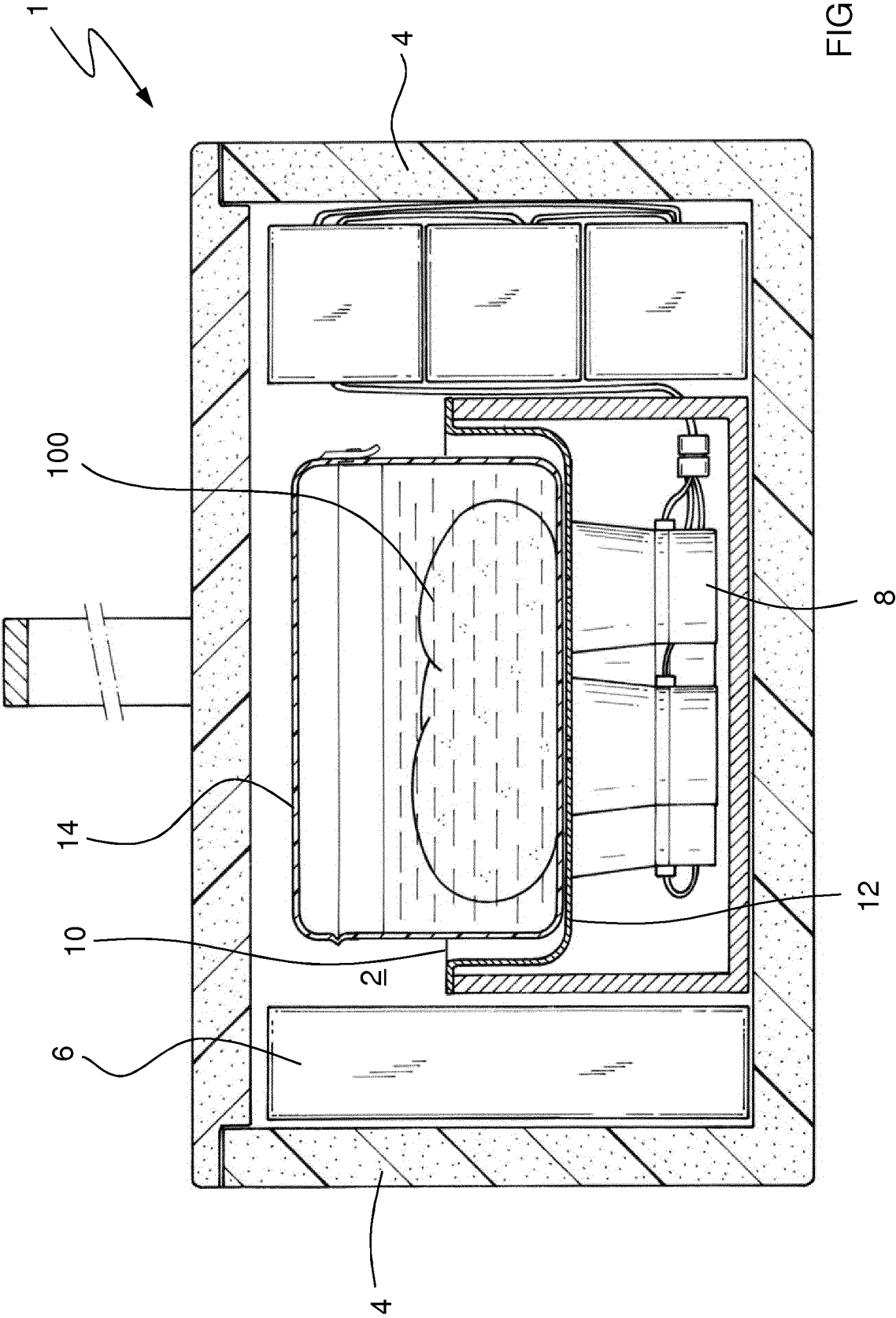


FIG. 7A

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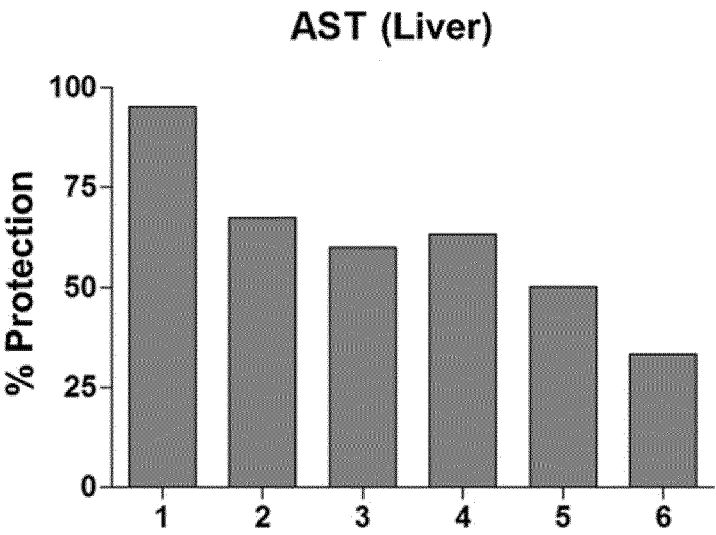


FIG.8A

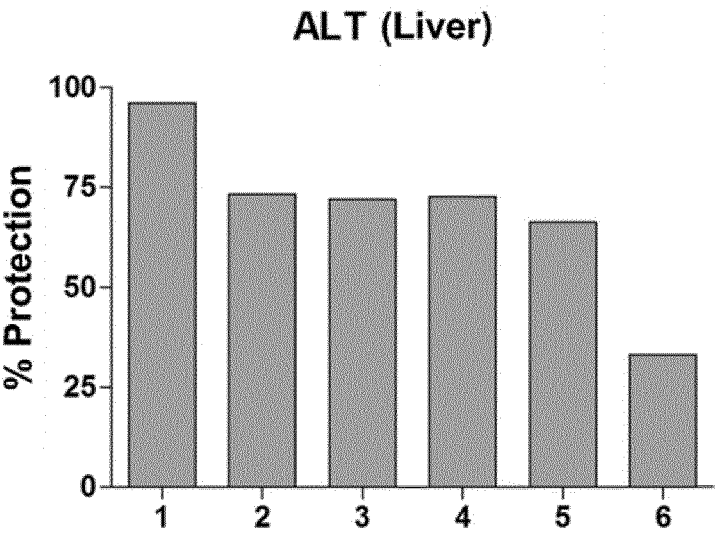


FIG.8B

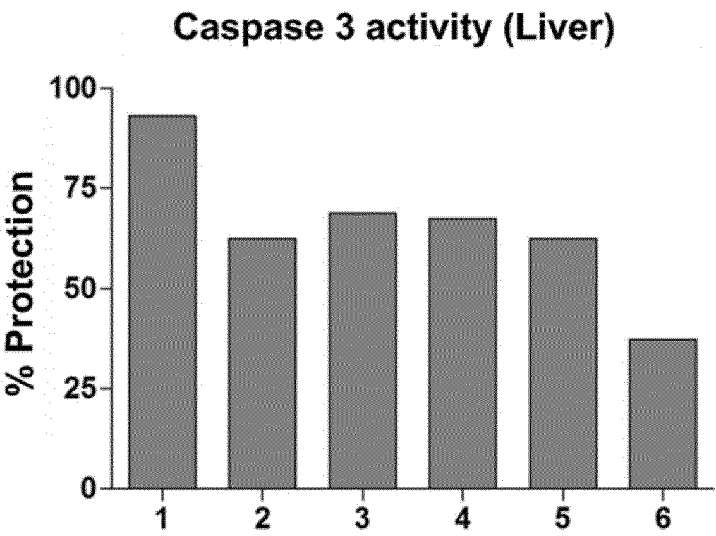


FIG.8C

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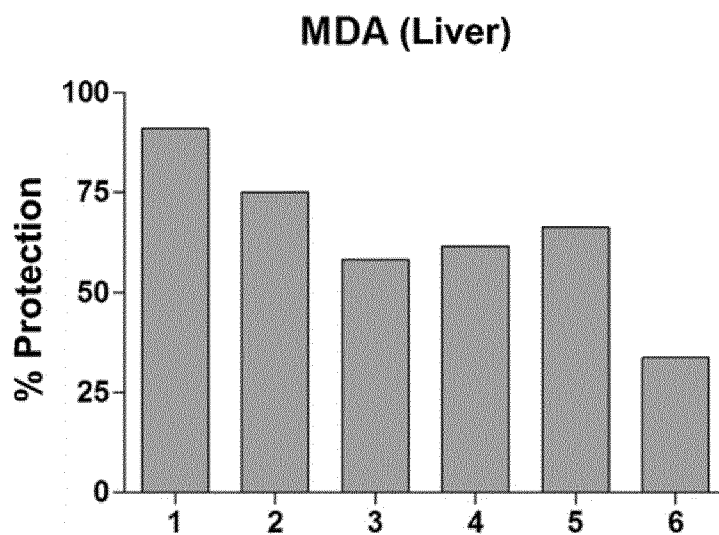


FIG.8D

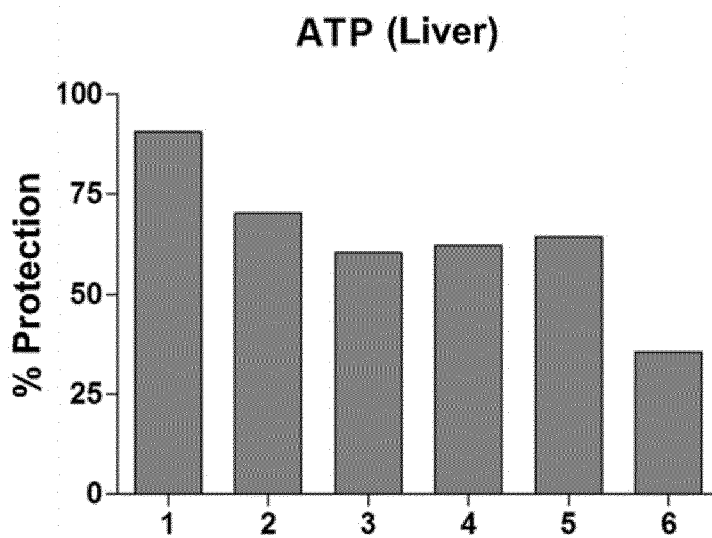


FIG.8E

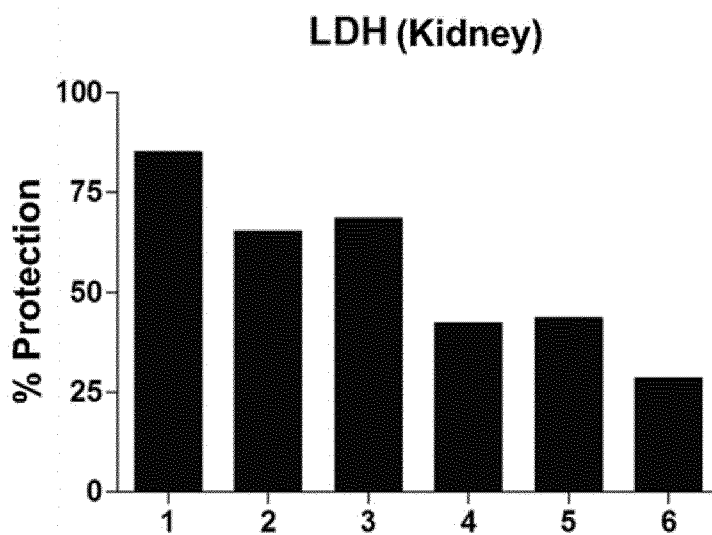


FIG.9A

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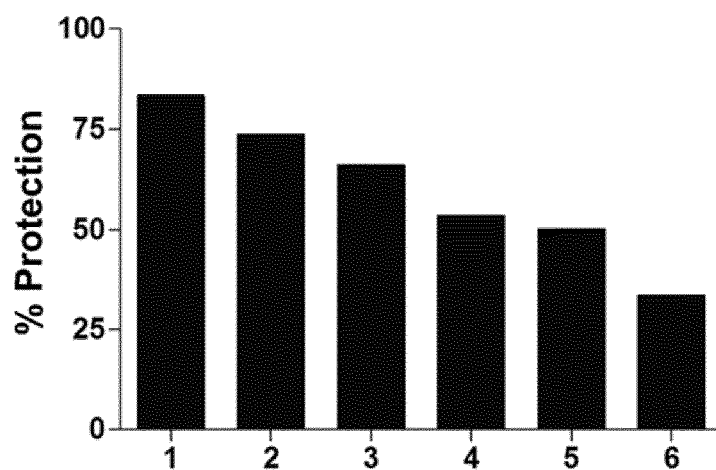
MDA (Kidney)

FIG.9B

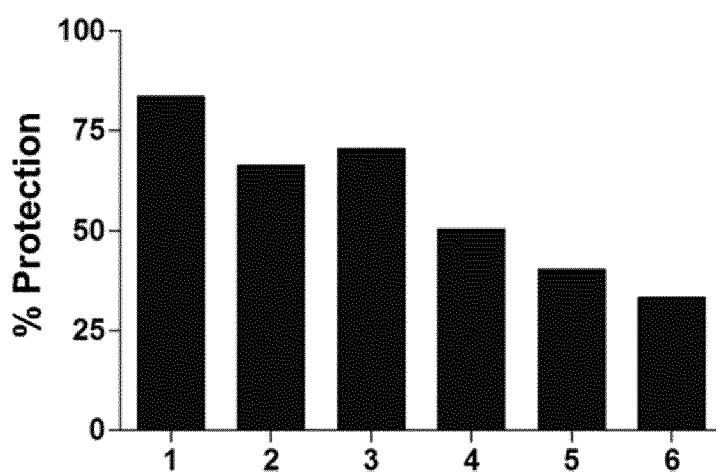
Caspase 3 activity (Kidney)

FIG.9C

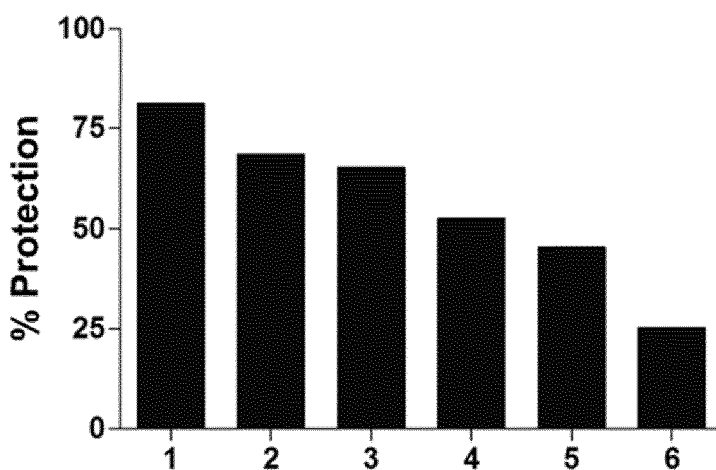
ATP (Kidney)

FIG.9D

Different preservation solutions AST (Liver)

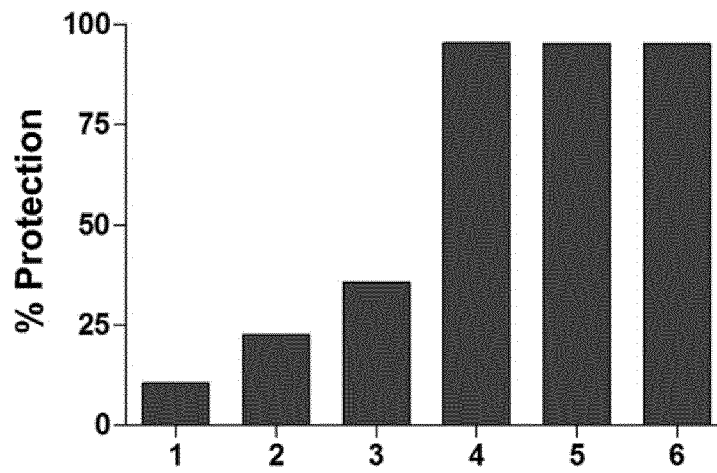


FIG.10

Different preservation solutions LDH (Kidney)

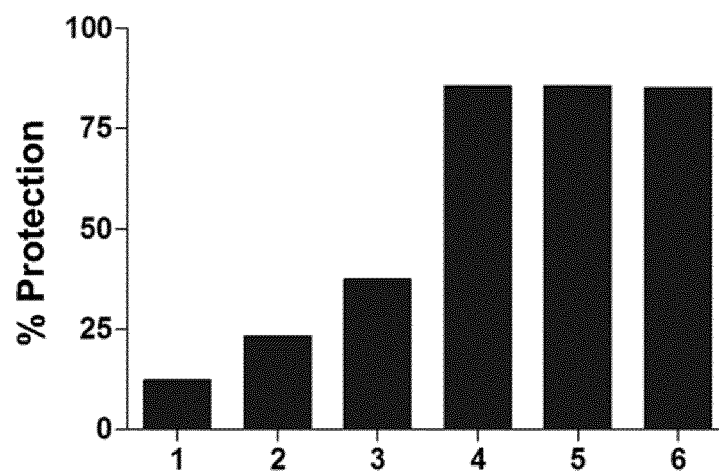


FIG.11

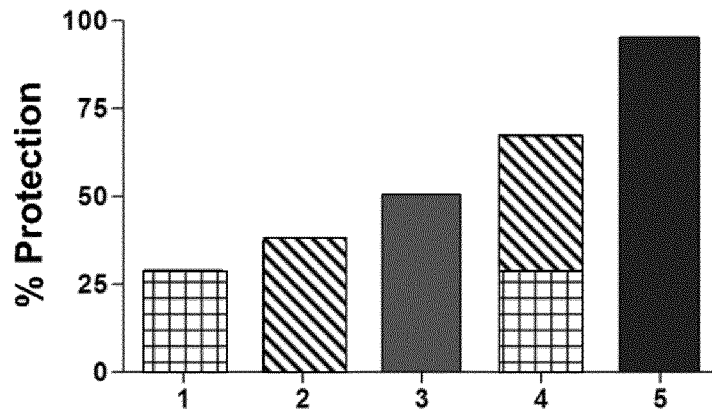
Expected vs. observed results AST (Liver)

FIG.12

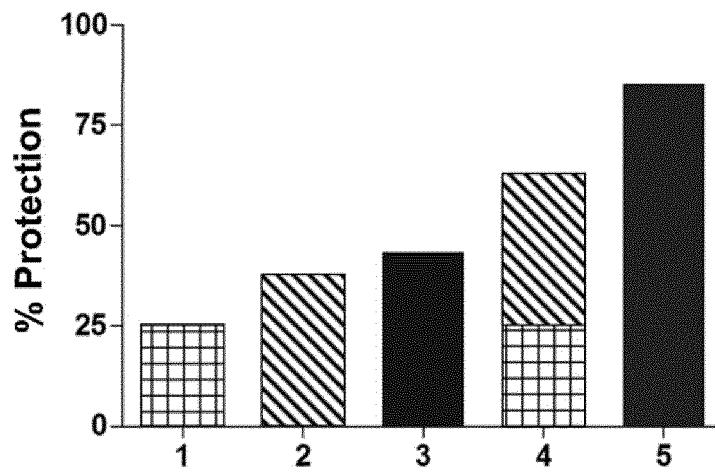
Expected vs. observed results AST (Kidney)

FIG.13