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(71) Applicant: UCL BUSINESS PLC [GB/GB]; The Network Building, 97 Tottenham Court Road, London, Greater London W1T 4TP (GB).

(72) Inventors: ROBERTSON, Nikki; Institute for Women's Health, 74 Huntley Street, London, Greater London WC1E 6HX (GB). OLIVER-TAYLOR, Aaron; Institute for Women's Health, 74 Huntley Street, London, Greater London WC1E 6HX (GB). ROSTAMI, Jamshid; Institute for Women's Health, 74 Huntley Street, London, Greater London WC1E 6HX (GB).

(74) Agents: KREMER, Simon et al.; Mewburn Ellis LLP, 33 Gutter Lane, London, Greater London EC2V 8AS (GB).

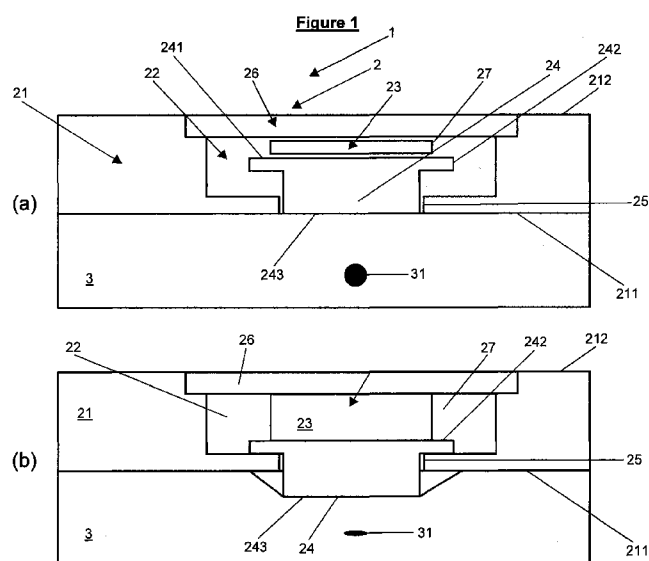
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(54) Title: DEVICE FOR REMOTE ISCHAEMIC CONDITIONING



(57) Abstract: Devices that allow remote ischemic conditioning to be carried out safely and efficiently are provided. Methods for performing remote ischemic conditioning on a subject are also disclosed.

DEVICE FOR REMOTE ISCHAEMIC CONDITIONING

Technical field

The present invention relates generally to methods and devices for remote ischaemic conditioning.

Background art

Ischaemia-reperfusion injury is caused by the restoration of blood supply to an organ after the occurrence of an ischaemic event.

Neonatal encephalopathy following perinatal hypoxic ischemic events (HIE) occurs in 1-3/1000 live births in high-income countries and 7-20/1000 live births in low and middle-income countries (Kurinczuk et al., 2010, Lawn et al., 2009). Perinatal hypoxic-ischaemic brain injury in the term baby therefore remains a significant problem throughout the world. Neonatal encephalopathy (NE) is the clinical manifestation of the ensuing disordered brain function. It is still responsible for nearly a quarter of 4 million annual neonatal deaths worldwide with devastating outcome (Lawn et al., 2005).

Serious consequences follow moderate to severe NE; these include death in 10-15%, cerebral palsy in 15% and other significant cognitive, developmental and behavioural problems in 40% of survivors. The financial and human costs to the family and society are thus very high. In the UK, half the medical litigation cases dealt with by the National Health Service Litigation Authority (NHSLA) relate to adverse events around birth, including NE and cerebral palsy. The outstanding liability of the NHSLA was £11.9 billion in 2008.

With a term birth rate in the UK of 750,000, the number of infants eligible for neuroprotection is 750-1125 per year. In low resource settings the incidence of neonatal encephalopathy is 10-15 times more common.

Therapeutic hypothermia is a safe and standard treatment for neonatal encephalopathy in developed countries (Jacobs et al., 2013) but its safety has not been studied in low and middle income countries. In the UK therapeutic hypothermia is used routinely – the number needed to treat (NNT) to prevent one adverse outcome is 6 for moderate and 7 for severe encephalopathy. In financial terms the total benefit to the UK economy with the introduction and uptake of therapeutic hypothermia is in excess of £1.25 million.

Therapeutic hypothermia reduces the combined rate of mortality and severe disability in moderate to severe HIE with NNT of 6-7 (Tagin et al., 2012). However still nearly 50 percent of cooled babies have an adverse neurological outcome (Edwards et al., 2010). Additionally, induced hypothermia was proven to be detrimental in meningitis induced encephalopathy (Mourvillier et al., 2013). Since cooling extends the therapeutic window (Fairchild et al., 2004, Liu et al., 2004, O'Brien et al., 2006), research is now being focused on pre-clinical and clinical studies for further novel and non-invasive hypothermia-augmented neuroprotective interventions (Kelen and Robertson, 2010, Robertson et al., 2012).

Recent studies have shown that combining other agents such as melatonin with cooling leads to improved outcomes compared to cooling alone. Furthermore, in some settings such as in parts of sub Saharan Africa, cooling is not standard therapy and simple, safe, economically viable and effective treatments to be used on their own are needed.

One simple protective intervention which harnesses the body's own intrinsic neuroprotection cascades is ischaemic pre- and post-conditioning. The concept of organ conditioning evolved following introduction of ischemia-reperfusion injury, or simply "reperfusion injury". Reperfusion injury is the main cause of cell injury and death following restoration of blood supply to a critically ischemic organ and was originally described in 1960 in the heart (Jennings et al., 1960). Its pathophysiology has been studied since then and vulnerability of microvasculature dysfunction of an ischemic organ is acknowledged as the key initiator of injury following reperfusion (Carden and Granger, 2000). Macrophage activation and circulating inflammatory mediators also cause additional microvascular injury in remote organs without preceding ischemia and cause multi organ dysfunction syndrome (Carden and Granger, 2000). Accordingly, protective conditioning strategies have been developed to limit ischemia-reperfusion injury.

Ischaemic preconditioning, and ischaemic postconditioning refer to the application of brief sublethal ischaemia in one organ before (preconditioning) or after (postconditioning) a prolonged injurious insult, generating tissue-protective mechanisms in the same organ. Ischaemic pre and post conditioning have been shown to be protective following ischaemia reperfusion in various organs including the myocardium and the brain. However, the application of ischaemic preconditioning is limited by the unpredictable nature of ischaemic insults in clinical practice.

Ischaemic postconditioning (IPostC) can be conducted *after* the ischaemic insult and therefore the timing is relevant to the treatment of perinatal asphyxia. The initial studies of IPostC were performed in the myocardium and consisted of interrupted reperfusion early in the reperfusion phase following an ischaemic insult. In animal models of stroke, IPostC has been shown to reduce cerebral infarct size in both focal (Zhao H *et al.*, 2006) and global (Wang J *et al.*, 2008) ischaemia and this is an important area of neuroprotection research (Zhao H *et al.*, 2009).

The beneficial effect size from ischaemic post conditioning is similar to that from therapeutic hypothermia; it is possible that ischaemic post conditioning could be used instead of therapeutic hypothermia in settings where cooling has not been shown to be safe.

Recent studies suggest that brief transient episodes of ischaemia are also effective if performed on a non-vital organ such as a limb remote to the affected organ. This forms the basis of *remote* IPostC (RIPostC) and is a much more feasible clinical treatment strategy because the ischaemic stimulus can be performed at a remote site (i.e. limb) that is easily accessible. RIPostC overcomes the obstacle of accessing the feeding artery of the ischemic organ in an emergency situation and the major drawback of applying further ischaemic stress on a vital organ after a major ischaemic event during cerebral postconditioning, which also has its own practical limitation. Rapid RIPostC when remote

ischemic stimulus is applied immediately or up to 30 minutes after cerebral reperfusion is proven to reduce infarct size and improve neurological function in a focal cerebral ischemic injury (Pignataro *et al.*, 2013, Qi *et al.*, 2012). Further, the therapeutic window is likely to be clinically feasible as RPostC is effective in improving brain metabolism, normalizing cerebral blood flow and providing long term neuroprotection if initiated as late as 3 or 6 hours after index ischaemia in stroke models (Ren C *et al.*, 2009). Importantly, this neuroprotective effect is also seen in models of *neonatal* hypoxic-ischaemic brain injury (Zhou Y FN *et al.*, 2011). RPostC therefore opens up an enormous translational potential for the newborn with perinatal asphyxia.

Postconditioning is typically achieved using inflatable blood pressure cuffs. These are positioned around the limbs, and inflated to occlude the arteries feeding the limb. However, it is difficult for these cuffs to ensure complete occlusion, as they do not specifically target the major feeding arteries. Furthermore there is the possibility of a crush injury, particularly on frail patients such as neonates.

WO 2012/142360 describes a system for performing remote ischaemic conditioning (RIC) which includes an inflatable cuff configured to encircle a limb. The cuff is inflated until blood flow occlusion pressure is achieved. Although this system is suitable to perform RIC, the pressure applied by the cuff could inflict injury such as crush injury to the subject, especially in neonatal subjects or subjects who have small or weak limbs to which the cuff is attached.

US 2010/0324429 refers to an RIC device which can be an inflatable cuff which is inflated when positioned on the limb of a subject in order to occlude blood flow through the limb. A device is also described including a single occlusion bulb held in place over the site of an arteriotomy, which may be inflated to perform ischaemic preconditioning before the procedure and also provide hemostasis and vascular sealing after the procedure. Such an inflatable bulb again cannot achieve targeted pressure on the specific artery and may cause pain or discomfort in tissues of the limb surrounding the artery.

There is currently no approved device for remote ischaemic postconditioning. There is therefore a need for improved devices and methods which offer the potential to carry out RIC, for example RPostC, in a safe, effective and efficient manner. This is especially necessary when considering the treatment of small subjects such as neonates.

Summary of the Invention

According to a first aspect of the invention, there is provided a device for remote ischemic conditioning including a body configured to attach to a limb of a subject, wherein the body comprises (i) a housing which provides an inner face for contacting the limb, (ii) a rigid arterial occlusion member which is reversibly extendible beyond the inner face of the housing, and (iii) an occlusion air bladder within the housing, wherein inflation of the occlusion air bladder causes the occlusion member to extend beyond the inner face of the housing.

The device allows remote ischaemic conditioning (RIC) to be carried out safely and efficiently. The position of the housing against the limb can be easily adjusted to achieve

accurate positioning of the occlusion member against the limb to achieve alignment with an artery within the limb. The rigidity of the occlusion member allows for localised pressure to be applied to a specific area of the limb corresponding to the location of the artery. High pressure does not need to be applied to surrounding areas of the limb. This reduces the overall load applied to the limb leading to a reduced risk of injury such as crush injury compared to existing devices which use inflatable cuffs.

Preferably, the device is adapted or configured to fit the anatomy of a neonate. This may include selecting a size of occlusion member suitable to occlude an artery within a limb of the neonate while avoiding unnecessary pressure to surrounding tissues. This may also include selecting the housing size and shape to correspond with the limb size and shape of the neonate. The inner face of the housing may be contoured to match the contour of a limb of the neonate, such as a leg.

Alternatively, the device may be adapted or configured to fit the anatomy of any other human subject.

Preferably, the device is disposable. The device may be intended for single use. This provides a hygienic device and ensures that subjects are protected from potentially unclean and contaminated devices.

Preferred devices of the present invention are adapted for controlled and repeatable occlusion and reperfusion.

Occlusion Member

The rigid occlusion member provides targeted occlusion of the artery which is impossible to achieve with known devices. The rigid occlusion member targets the artery directly rather than relying on the compression of surrounding tissue. It is difficult to achieve complete occlusion of the artery using known inflatable cuff devices and devices which use non-rigid occlusion members, because the pressure applied is spread over a much greater area of the limb and therefore a greater overall pressure must be applied to achieve the same degree of occlusion.

In subjects with very short limbs, such as neonatal subjects, it is difficult to use standard devices such as inflatable cuffs to provide arterial occlusion, since these are often wide cuffs which cannot concentrate pressure on a particular area of the small limb. Furthermore, because these known cuffs operate by constricting the limb from all sides, the internal diameter of the cuff when inflated must be very small to achieve arterial occlusion in a neonate. In comparison to a cuff, the device of the present invention allows for maximum ischaemia of the entire limb, and therefore the maximum post conditioning effect.

Preferably, the body is configured to attach to a leg of a subject such that the arterial occlusion member overlies and acts upon the femoral artery to occlude the femoral artery when the occlusion air bladder is inflated.

The femoral artery in particular presents problems when attempting occlusion. The femoral artery is very close to its branches in small subjects such as neonates, and devices which fail to target the femoral artery alone can result in an unsafe ischaemia procedure with unwanted secondary effects on tissues surrounding the femoral artery. The present device can easily target a specific artery.

Balloon catheters are often used to induce ischaemia within a limb (EP 2 353 501 A1). The present device provides a non-invasive alternative to such devices with the advantages described herein.

In general terms, the device according to the present invention achieves complete arterial occlusion in a highly effective way, leading to improved RIC treatment. Furthermore, because the occlusion member is retractable within the housing the device may be left in place against the limb of the subject throughout the RIC procedure.

The occlusion member may be made from any suitable rigid material. Preferably, the rigid occlusion member is moulded from plastics material. Alternatively, the rigid occlusion member may be produced by a 3D printing method such as those described in US 2013/0176312 A1, the disclosure of which is incorporated herein by cross reference in its entirety.

Preferably, the occlusion member comprises a contact surface intended to be brought into contact with the limb when the occlusion member is in an extended position. Preferably, the contact surface when the occlusion member is in a fully retracted position has a contour which matches and is a continuation of the contour of the inner surface of the housing. Preferably, the contact surface is convex.

Preferably, the occlusion member comprises an abutment surface which abuts the housing when the occlusion member reaches a fully retracted position, such that the occlusion member cannot retract further and a substantially continuous contour defined by the contact surface of the occlusion member and the inner surface of the housing is presented.

Preferably, the occlusion member has a length of at least 2mm, 3mm, 4mm, 5mm, 6mm, 7mm, 8mm, 9mm or 10mm. Preferably, the occlusion member has a length of up to 50mm, 40mm, 30mm, 20mm or 15mm. More preferably, the occlusion member has a length of from 20mm to 30mm. The length may be about 24mm.

Preferably, the occlusion member has a width of at least 2mm, 3mm, 4mm, 5mm, 6mm, 7mm, 8mm, 9mm or 10mm. Preferably, the occlusion member has a width of up to 50mm, 40mm, 30mm, 20mm or 15mm. More preferably, the occlusion member has a width of 4mm to 6mm. The width of the occlusion member may be about 5mm.

The length and width of the occlusion member define the contact surface area of the occlusion member which contacts the limb and lies in a plane substantially parallel with the inner face of the housing.

Where the occlusion member includes a collar or flange which acts as a retaining means, the collar or flange may be longer than the length of the occlusion member by about 10mm. The collar or flange may be wider than the width of the occlusion member by about 1-2mm. The collar or flange may be about 1-2mm thick.

Preferably, the occlusion member is extendable from the inner face of the housing by up to 50mm, 40mm, 30mm, 20mm, 10mm or 5mm. More preferably, the occlusion member is extendable from the inner face by 5mm-35mm. The minimum distance that the occlusion member is required to extend is defined by the depth of the artery within the limb. This will depend on the nature of the subject, and will be lower in e.g. neonatal subjects, but may be at least 1, 2, 3, 4, or 5mm for example.

Preferably, when the device is adapted or configured to fit the anatomy of a neonate the occlusion member is extendable from the inner face of the housing by up to 30mm, 25mm, 20mm, 15mm, 10mm or 5mm.

Air Bladder

The occlusion air bladder within the housing of the device provides control over the extension of the occlusion member. The degree of inflation dictates the degree of extension of the occlusion member out of the housing and thereby the degree of occlusion of the artery within the limb. By exercising control over the inflation of the air bladder the degree of occlusion of the artery can be controlled very accurately which is crucial to the successful performance of RIC. Existing devices which use an air bladder encircling the limb offer no such accuracy and do not provide the user of the device with control over the degree of arterial occlusion.

The air bladder may be made from any suitable material by any suitable method. The air bladder is preferably similar to air bladders already well known and used in blood pressure cuffs and similar devices, for example the bladder described in WO 2012/142360 A2, the disclosure of which is incorporated herein by cross reference in its entirety. The air bladder may be intended for single use only.

The air bladder preferably includes a valve to facilitate inflation and deflation.

The arterial occlusion member is reversibly extendible beyond the inner face of the housing. Extension of the occlusion member out of the housing is achieved by inflating the air bladder. When the air bladder is deflated, the occlusion member will retract back within the housing simply due to its contact with the limb. No biasing means are necessary to retract the occluding member within the housing, although such biasing means may optionally be included if desired.

The construction of preferred devices according to the present invention provides them with MRI system compatibility. The device can therefore be safely used within an MRI system which can gather data on the subject while the RIC is performed. The avoidance of the use of metal in any part of the appliance makes the device MRI compatible and thus preferred devices will have an all-plastic housing and pneumatic actuation. Existing devices such as blood pressure cuffs or other systems may have metallic components

which would not provide MRI compatibility. Devices such as those described in WO 2012/142360 include an on-board controller and are therefore not MRI compatible.

Preferably, the device comprises an auxiliary air bladder outside the housing and operable independently from the occlusion air bladder, wherein the auxiliary air bladder is configured to contact the limb such that inflation of the auxiliary air bladder tightens the housing of the device against the limb.

The auxiliary air bladder may be inflated at the same time as the occlusion air bladder within the housing. When fully inflated, the auxiliary bladder helps to ensure firm contact of the device housing with the limb. The auxiliary bladder may remain inflated for the duration of RIC and fine adjustment of arterial occlusion may then be performed by adjusting the degree of inflation of the air bladder within the housing independently. The auxiliary air bladder may be positioned against a portion of the limb opposing the position of the device housing. Preferably, the auxiliary air bladder is not itself used to occlude any blood vessels within the limb when inflated. The pressure applied by the auxiliary air bladder may be enough to ensure firm contact of the device housing with the limb while not being sufficient to cause any arterial occlusion within the limb. This allows the arterial occlusion member to target the artery without interference from the auxiliary air bladder.

RIC consists of cycles including periods of ischaemia (the occlusion or part occlusion of a blood vessel to restrict the flow of blood to a limb remote from the ischaemic event) and reperfusion (the restoration of blood flow to the limb) which are repeated a number of times. For example, a typical RIC protocol may include 4 cycles consisting of a series of 4 periods of ischaemia (lasting 10 minutes) and reperfusion (lasting 10 minutes). The auxiliary air bladder provides improved occlusion during the ischaemia periods and ensures unrestricted blood flow during the intervening reperfusion periods. This provides an improved RIC protocol compared with existing devices, which cannot provide both the complete occlusion and unrestricted blood flow necessary for successful RIC.

Preferably, the body is configured to attach to the leg of a subject such that the arterial occlusion member overlies and acts upon the femoral artery to occlude the femoral artery when the air bladder is inflated. Occlusion of the femoral artery is advantageous because there is more muscle mass in the lower than upper limbs. The therapeutic post conditioning effect increases with the mass of tissue that remote ischaemia is performed upon.

Preferably, the femoral artery is occluded within the inguinal crease. This will induce ischaemia in the greatest possible mass of the leg and will therefore provide the greatest therapeutic effect. The configuration of existing devices which include cuffs cannot access the inguinal crease of a subject. They are therefore limited to application lower down the limb and the mass of tissue in which ischaemia can be applied is reduced. In the present case, preferred devices are adapted or configured to sit within the inguinal crease. The femoral artery can therefore be targeted at the base of the limb and maximum limb ischaemia is achieved.

The femoral artery supplies blood to the thigh, and is relatively exposed within the inguinal crease. Occlusion of this artery provides sub-lethal ischaemia in the leg (a non-vital organ) which triggers the body's intrinsic neuroprotection cascades to treat the effects of an ischaemic insult.

Alternatively, the body may be configured to attach to another non-vital organ of the subject. This may be the arm (in order to occlude e.g. the brachial artery).

Device Housing

Preferably, the housing defines (i) a chamber containing the occlusion air bladder and at least part of the retracted occlusion member, and (ii) an opening in the inner face through which the occlusion member can be reversibly extended.

Such an arrangement allows for a compact device, which is highly advantageous for devices intended for the performance of RIC on neonatal subjects. Furthermore, containing the air bladder within a chamber allows its inflation to be concentrated on the occlusion member, which means that less gas will need to be supplied to the bladder in order to extend the occlusion member by a given amount, and the device will therefore operate more efficiently.

Preferably, the housing comprises an outer face substantially parallel with the inner face. Preferably, the inner and outer faces are separated by and lie substantially perpendicular to two side faces and two end faces. The housing therefore preferably comprises six faces - an outer face and an inner face opposite one another, two side faces opposite one another and two end faces opposite one another, each of the three pairs being substantially mutually perpendicular.

Preferably, the housing is made from plastics material. Preferably, the device is manufactured by injection moulding, or similar manufacturing process known to those skilled in the art. Alternatively, the housing may be made of nylon. The housing may be produced by a 3D printing process such as those described in US 2013/0176312 A1, the disclosure of which is incorporated herein by cross reference in its entirety. The housing is preferably one integral piece. Preferably, a hatch is provided which may be a separate piece to the housing.

Preferably, the occlusion member includes retaining means for retaining at least part of the occlusion member within the housing.

The retaining means limit the advancement of the occlusion member out of the housing. This provides a safety mechanism by providing a terminal position for the extension of the occlusion member. The retaining means will prevent further extension of the occlusion member beyond a given position even after further inflation of the air bladder within the housing. This means that accidental injury to the subject by over-inflation of the air bladder may be prevented. The retaining means may be configured to allow the occlusion member to extend to a position which allows for complete occlusion of the artery, but to terminate the extension before unnecessary injury occurs. Existing devices

which use inflatable cuffs or inflatable occlusion members provide no way to prevent over-compression of the limb if the air bladder is over-inflated.

Furthermore, the retaining means ensure that the occlusion member is retained securely within the housing so that it can perform its function during RIC.

Preferably, the retaining means is a portion or collar of the occlusion member which is larger than the opening in the inner face of the housing and is retained within the chamber when the air bladder is inflated.

Such a retaining means allows for a simple device construction and easy assembly of the component parts. Alternatively, the retaining means may comprise a connector between the occlusion member and the housing which is placed under tension when the occlusion member is in a fully extended position to preclude further advancement of the occlusion member upon further inflation of the air bladder.

Preferably, the body comprises a hatch to provide access to the housing interior.

If the user of the device has easy access to the interior of the housing, maintenance of the device is made easy. The component parts such as the occlusion member and air bladder are easily accessible and can be checked for problems before the device is used for RIC. The air bladder and occlusion member may be easily replaced if required. If a single use air bladder is used in the device, the housing interior is easily accessible so that the air bladder may be replaced before the next ischaemia/reperfusion cycle is carried out.

Preferably, the hatch is a removable panel reversibly attached to an outer face of the housing. In this way, the air bladder is located within the housing between the hatch and the occlusion member and is more easily accessible. The hatch may be a screw-fit or push-fit cap. Preferably, the removable hatch or panel is secured to the housing using snap-fit moulded plastic fittings. More preferably, the removable panel is attached to the housing by a living hinge. This arrangement enables the entire body (housing and panel) to be manufactured as a single piece, preferably by injection moulding. Alternatively, the panel is secured to the housing with nylon screws.

Preferably, the inner face of the housing has one or more rounded or bevelled edges. When the housing is secured against the limb of a subject, such rounded or bevelled edges provide a comfortable arrangement and allow the device to be securely fixed against the limb with minimal pain or discomfort.

Preferably, in lateral section, or profile, the front of the body or housing may substantially define (i) a square or rectangle; (ii) rounded-corner square or rectangle (or 'squircle'); (iii) an arch having parallel lines on the inner and outer faces of the housing and curvature (e.g. half or quarter circle curvature) at one side face and either an open or straight profile at the other side face; or (iii) a stadium having parallel lines on the inner and outer faces of the housing and curvature (e.g. half or quarter circle curvature) on the left and right side faces.

One or both sides of the housing may be narrowed or bevelled or otherwise curved, to permit greater accuracy of manipulation of the artery if desired.

The inner face of the housing is preferably convex.

Preferably, the length of the housing is at least 20mm, 30mm, 40mm or 50mm. Preferably, the length of the housing is up to 200mm, 180mm, 160mm, 140mm, 120mm or 100mm. More preferably, the length of the housing is 70mm to 90mm. The length of the housing may be 80mm.

Preferably, when the device is adapted or configured to fit the anatomy of a neonate the length of the housing is at least 20mm, 25mm, 30mm or 40mm. Preferably, when the device is adapted or configured to fit the anatomy of a neonate the length of the housing is up to 100mm, 90mm, 80mm, 70mm or 60mm.

Preferably, the width of the housing is at least 5mm, 10mm, 15mm or 20mm. Preferably, the width of the housing is up to 100mm, 90mm, 80mm, 70mm, 60mm or 50mm. More preferably, the width of the housing is from 10mm to 20mm. The width of the housing may be 15mm.

Preferably, when the device is adapted or configured to fit the anatomy of a neonate the width of the housing is at least 10mm, 15mm, 20mm or 25mm. Preferably, when the device is adapted or configured to fit the anatomy of a neonate the width of the housing is up to 60mm, 50mm, 40mm or 30mm.

The length and width of the housing define the inner and outer face areas of the housing. The length and width therefore define the area which will be in contact with a limb during the RIC procedure.

Preferably, the depth of the housing is at least 2mm, 4mm, 6mm or 8mm. Preferably, the depth of the housing is up to 70mm, 60mm or 50mm. More preferably, the depth of the housing is from 10mm to 20mm. The depth of the housing may be 15mm. The depth of the housing is the length of an edge of the housing perpendicular to the inner face.

Preferably, the housing has a thickness of at least 0.5mm. Preferably, the housing has a thickness of up to 10mm, 9mm, 8mm or 7mm. More preferably, the housing thickness is from 1mm to 6mm.

The thickness may be uniform over the entire housing. Alternatively, the thickness may vary and thicker housing may be used for certain parts of the housing to provide greater strength.

Attachment Means

Preferably, the body comprises attachment means for securing the device to the limb of the subject. The attachment means allow the device to be fixed into the correct position against the limb so that occlusion of the artery is possible by extension of the occlusion

member. The attachment means also provide the necessary tension of the housing against the limb so that extension of the occlusion member can successfully occlude the artery. Together with the auxiliary air bladder, this provides an efficient and effective device for performing RIC.

Preferably, the attachment means is a belt, strap or clamp configured to encircle or grasp the limb. This allows for easy adjustment of the tension in the attachment means and the pressure applied to the limb by the housing before RIC is carried out. The attachment means may be a hook-and-loop strap which passes around the limb and secures the housing against the limb. Because of the use of the separate occlusion member, the pressure applied to the limb by the attachment means will be lower than the pressure applied by known devices such as inflatable cuffs. This reduces the risk of crushing injury and reduces the discomfort and pain experienced by the subject during the RIC process.

Preferably, the attachment means is selected to fit the anatomy of a neonate. This may include selecting a belt or strap of appropriate length and width to provide a comfortable and suitable means of attaching the housing to the limb of a neonate.

Multi-body Device

Preferably, the device includes two or more bodies as defined in any one of the preceding claims, each body being configured to attach to a different limb of the subject. As discussed above, the larger the mass of tissue that remote ischaemia is performed upon, the greater the therapeutic effect. Therefore, performing remote ischaemia on multiple limbs provides an increased therapeutic effect over a device which performs ischaemia on only one limb.

The device may include two bodies flexibly or rigidly attached to each other. The bodies may each be configured to attach to a different leg of the subject. The bodies may each be configured to attach to a different arm of the subject. The bodies may be configured such that a first body attaches to a leg of the subject and a second body attaches to an arm of the subject.

The device may include four bodies, two of which being configured to attach to a different leg of the subject and two of which being configured to attach to a different arm of the subject.

Preferably, the device includes two bodies connected to each other by a hinge and each configured to attach to a different leg of the subject.

The two bodies may be arranged in a configuration which allows each body to sit securely along an inguinal canal of the subject. This may be a "V" shaped configuration, wherein each body comprises an elongate housing and the apex of the "V" is defined where the two housings meet. The two bodies are preferably connected by a hinge at the point of connection. The angle of the "V" shape defined by the two bodies can be varied by rotating about the hinge. The angle is preferably adjusted to provide a device configuration suited to the anatomy of the subject.

When such a "V" shape is provided, the device is preconfigured to lie in the correct anatomical position to occlude the artery and the positioning procedure is simplified, allowing it to be performed easily without any extensive training or guidance. The device then naturally sits over the artery. It may for example sit naturally in the inguinal crease and the positioning and size of the occlusion member means that the occluding member will naturally be positioned over the femoral artery.

When attached to the legs of the subject, a first housing will lie along the right inguinal crease and a second housing will lie along the left inguinal crease. The apex of the "V" will point towards the peripheral region of the subject (i.e. the feet). Each occlusion member will be positioned so as to occlude a femoral artery when extended, so that both right and left femoral arteries may be occluded during RIC. Each housing may include its own individual attachment means for attachment to a respective leg, or alternatively a single attachment means may be provided for the composite device.

Gas Supply System

Preferably, the device comprises a gas supply system connected to the occlusion air bladder and/or the auxiliary air bladder. A gas supply system allows for controlled inflation and deflation of the air bladder.

Preferably, the gas supply system includes (i) a gas source, (ii) an inlet valve downstream of the source, (iii) an exhaust valve, and (iv) a gas reservoir.

Preferably, the gas source is a compressed gas source. Alternatively, the gas source may comprise a motor and compressor.

Preferably, the compressed gas source is a compressed gas cylinder. The cylinder may be attached to a regulator and pressure gauge. The compressed gas may be nitrogen gas. Alternatively, the compressed gas may be compressed air. The compressed gas source is preferably controllable to supply the air bladder with the necessary amount of gas to achieve the required degree of inflation.

The gas supply system preferably includes a gas line to connect each of the components. This may be a flexible or rigid gas line. The gas line may be fixed or adjustable.

The inlet valve is preferably a solenoid valve. Alternatively, the inlet valve may be a ball valve or needle valve. Alternatively, any other suitable gas line valve known to those skilled in the art may be used. The exhaust valve is preferably a solenoid valve. Alternatively, the exhaust valve may be a ball valve or needle valve. Alternatively, any other suitable gas line valve known to those skilled in the art may be used. The inlet valve is preferably operable to control the rate of inflation and the exhaust valve is operable to control the rate of deflation. The air reservoir may assist in fine-tuning the pressure of the air bladder since the system will be provided with a greater overall internal volume.

The inlet valve is preferably downstream of the gas source. The exhaust valve is preferably downstream of the inlet valve. The gas reservoir is preferably downstream of the inlet valve. The exhaust valve is preferably downstream of the gas reservoir.

Where an auxiliary air bladder is present in the device, the gas supply system preferably includes separate gas lines to supply the occlusion air bladder and the auxiliary air bladder. These separate gas lines may be connected to the same compressed gas source but independently operable by different inlet valves. Alternatively, both the occlusion air bladder and the auxiliary air bladder may be supplied by the same gas line.

Control System

Preferably, the device comprises a control system for controlling the inflation of the air bladder to achieve the required arterial occlusion. The control system provides a consistent inflation pressure during the ischaemia periods, which is important to ensure that the necessary arterial occlusion is maintained. The control system ensures that the air bladders remain deflated at all other times, such as during reperfusion periods. The control system can detect and respond to any changes in the air bladder inflation pressure. If the pressure falls and there is a risk that arterial occlusion will be incomplete, the control system can correct the pressure to ensure successful occlusion. If the pressure rises above the level necessary to achieve complete occlusion, there may be a risk of injury to the subject and the device will be less efficient. The control system can ensure that the pressure threshold for occlusion is not exceeded.

Preferably, the control system includes (i) a gas source, (ii) a gas supply pressure sensor connected to the gas source, (iii) an inlet valve downstream of the gas supply pressure sensor, (iv) a device pressure sensor downstream of the inlet valve, (v) an exhaust valve, (vi) a gas reservoir, and (vii) a microcontroller connected to the pressure sensors and valves.

Preferably, the gas source is a compressed gas source. Alternatively, the gas source may comprise a motor and compressor.

Such a system can be fully automated. The microcontroller continuously monitors gas supply pressure and air bladder pressure. The microcontroller provides feedback based on the pressure levels detected to adjust the inlet and exhaust valves as necessary to achieve the required air bladder inflation. The inlet valve gates the inflow of gas from the gas supply for inflation. The exhaust valve provides an exhaust to control deflation.

A control system which may be useful in this regard is described in EP 2 353 501 A1, which is incorporated herein by cross-reference in its entirety.

During ischaemia periods the air bladder pressure may be maintained at a value of at least 10kPa, 20kPa, 30kPa or 40 kPa. During ischaemia periods the air bladder pressure may be maintained at a value of up to 100 kPa. More preferably, the air bladder pressure is maintained at 35-45 kPa during ischaemia periods. The air bladder pressure is most preferably maintained at about 40 kPa during ischaemia periods.

Increased pressure provides more effective occlusion but also decreases the lifespan of the air bladders. A pressure of around 40 kPa provides a good balance between achieving sufficient occlusion and a satisfactory lifespan of the air bladders.

The necessary pressure will depend on the exact size, shape and type of air bladder used. The skilled person will easily be able to determine a suitable air bladder pressure.

The pressure levels detected by the microcontroller may be displayed on a suitable interface so that a user of the device can monitor the process. The interface may be a screen or monitor. The interface may be an LCD screen. The interface may also provide the user with additional data relating to the RIC procedure. The additional data may include the time elapsed since the previous ischaemia or reperfusion period ended, or the time remaining in the current ischaemia or reperfusion period.

The control system may also display to the user an analogue voltage proportional to the pressure of the air bladder within the RIC device. The control system may also display a digital gating signal which may be high when the bladder is inflated. These may be provided at BNC sockets alongside the display medium so that they are easily accessible to the user.

The air bladder pressure sensor is preferably an electronic sensor.

The control system may be MRI compatible. This will allow the entire device to be kept within the MRI scan room. To achieve this, the control system may include components similar to non-invasive MRI compatible blood pressure monitors, such as the TeslaNIBP (RTM) manufactured by Mammendorfer Institut Fur Physik Und Medizin (MIPM).

Remote Ischaemic Conditioning Method

According to a second aspect, the present invention provides a method of performing remote ischaemic conditioning on a subject, including the steps of (a) attaching a device as defined according to the first aspect to a limb of the subject, (b) inflating the air bladder and maintaining inflation at a predetermined pressure for a predetermined period of time to cause the occlusion member to extend beyond the inner face of the housing and apply pressure to the limb, thereby occluding an artery within the limb, (c) deflating the air bladder and maintaining deflation for a predetermined period of time to retract the occlusion member, and (d) optionally repeating steps (b) and (c) a predetermined number of times to complete the remote ischaemic conditioning.

Preferably, the device is attached to a leg of the subject. This provides the device with access to the femoral artery. As described above this is advantageous due to the increased tissue mass subjected to RIC.

Inflation in step (b) may be maintained for a period of at least 30 seconds, 40 seconds, 50 seconds or 60 seconds. Inflation in step (b) may be maintained for a period of up to 20 mins, 18 mins, 16 mins, 14 mins, 12 mins, 11mins, 10 mins, 9 mins or 8 mins. Preferably, inflation in step (b) is maintained for 8-12 mins. If the inflation in step (b) is maintained for an excessive period of time, irreversible ischaemia may occur.

Deflation in step (c) may be maintained for a period of at least 30 seconds, 40 seconds, 50 seconds or 60 seconds. Deflation in step (c) may be maintained for a period of up to 20 mins, 18 mins, 16 mins, 14 mins, 12 mins, 11mins, 10 mins, 9 mins or 8 mins. Preferably, inflation in step (b) is maintained for 8-12 mins.

The inflation period in step (b) may be about 10 minutes. The deflation period in step (c) may be about 10 minutes.

Steps (b) and (c) may be performed once, to provide one ischaemia/reperfusion cycle. Preferably, the steps are repeated at least twice. More preferably, the steps are repeated at least three times. Preferably, steps (b) and (c) are performed up to a maximum of 10 times.

Preferably, the remote ischaemic conditioning is performed after the occurrence of an ischaemic event in the subject. In other words, the method preferably provides remote ischaemic post conditioning (RIPostC). The method can therefore be used to treat the aftermath of an ischaemic event. The method is therefore relevant to the treatment of perinatal asphyxia.

Alternatively, the remote ischaemic conditioning may be performed before the occurrence of an ischaemic event in the subject (RIPreC). The remote ischaemic preconditioning may be carried out prior to a surgical intervention. This will offer protection from ischaemic insults occurring during surgery. More preferably, the remote ischaemic preconditioning is carried out prior to infant cardiac surgery.

Preferably, the subject is a neonate and the remote ischaemic conditioning is performed to treat perinatal asphyxia. Neonatal encephalopathy due to perinatal asphyxia leads to high mortality with lifelong chronic disabilities, which can be mitigated or avoided by applying the method of the present invention. Alternatively, the subject may be any human or animal subject in need of treatment or protection.

Preferably, the device is attached to a leg of the subject and the artery occluded in step (b) is the femoral artery.

Preferably, during steps (b), (c) and (d) blood flow within the occluded limb is monitored. This may be carried out by monitoring asphygmia and sphygmus upon inflation and deflation of the air bladder. The monitoring may be carried out by the use of one or more pulse oximeters. Alternatively, the monitoring may be carried out by laser Doppler velocimetry.

Body for Remote Ischaemic Conditioning Device

According to a third aspect, there is provided a body for use in the device according to the first aspect, configured to attach to a limb of a subject, wherein the body comprises (i) a housing which provides an inner face for contacting the limb, (ii) a rigid arterial occlusion member which is reversibly extendible beyond the inner face of the housing, and (iii) an

air bladder within the housing, wherein inflation of the bladder causes the occlusion member to extend beyond the inner face of the housing.

Brief Description of the Drawings

Fig. 1 is a cross section through the body of a device according to the present invention.

Fig. 2 shows perspective views of the body of a device according to the present invention.

Fig. 3 shows a device according to the present invention.

Fig. 4 shows a gas supply and control system of a device according to the present invention.

Fig. 5 shows a plot of NTP/EPP against time elapsed during induced global hypoxic and cerebral ischaemic insult of a piglet.

Fig. 6 is a graphical representation of the procedure carried out on piglet models for perinatal asphyxia.

Fig. 7 shows a device according to the present invention attached to the hind limbs of a piglet.

Fig. 8 shows laser Doppler skin perfusion measurements of blood flow in the piglet model alongside a trace of device air bladder pressure.

Fig. 9 shows plots of (a) Lac/NAA white matter ratio against time; (b) Log(Lac/NAA thalamus ratio) against time; and (c) NTP/ePP whole brain ratio against time for both the control group and the RIPostC group.

Fig. 10 shows plots of (g) oxygen saturation; (h) heart rate, and (i) mean arterial blood pressure for both the control group and the RIPostC group.

Fig. 11 shows plots of changes in the concentration of (a) oxidised Cytochrome oxidase (b) cerebral blood volume, and (c) brain oxygenation for both the control group and the RIPostC group.

Fig. 12 shows the mean TUNEL counts in both the control group and the RIPostC group in eight studied regions.

Fig. 13 shows the results of TUNEL assessment of both the control group and the RIPostC group.

Fig. 14 shows the results of mean IBA-1 scores for both the control group and the RIPostC group.

Fig. 15 shows the assessment of CART and ABCC9 genes in both the control group and the RIPostC group.

Fig. 16 shows the results of western blots for Akt, Erk and Tubulin in both the control group and the RPostC group.

Fig. 17 shows the 9.4 Tesla Agilent MR scanner used to obtain ^1H and ^{31}P magnetic resonance spectra.

Fig. 18 shows a detailed dimensional drawing of a device according to one embodiment of the present invention, produced using CAD software. Shown are (a) side view; (b) lateral cross section through housing and occlusion member; (c) plan view showing outer face of housing; (d) longitudinal cross section through housing and occlusion member; and (e) side view.

Detailed Disclosure of the Invention

Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

The disclosure of all references cited herein, inasmuch as it may be used by those skilled in the art to carry out the invention, is hereby specifically incorporated herein by cross-reference.

Definitions

“RIC” refers to Remote Ischaemic Conditioning.

“RPostC” refers to Remote Ischaemic Postconditioning. RPostC is the protective effect elicited in one organ from the application in another organ of several brief intermittent episodes of ischaemia at the onset of reperfusion after severe hypoxia-ischaemia (HI).

“IBA1” refers to ionized calcium binding adaptor molecule 1.

“MRS” refers to magnetic resonance spectroscopy.

“NTP” refers to nucleotide triphosphate.

“EPP” refers to exchangeable phosphate pool.

“TUNEL” refers to transferase-mediated deoxyuridine triphosphate nick-end labelling.

“NIRS” refers to near-infrared spectroscopy.

Specific Embodiments of the Invention

Fig. 1 shows cross sections through one embodiment of the device 1 according to the present invention. Fig. 1a shows the device in the inflated state (to achieve occlusion in

the ischaemia periods). Fig. 1b shows the device in the deflated state (to allow blood flow in reperfusion periods).

The device 1 includes body 2. The body 2 comprises a housing 21 which defines a chamber 22. Housing 21 includes an inner face 211 and an outer face 212. Within the chamber 22 there is an air bladder 23 which is shown in a deflated state in Fig. 1a and in an inflated state in Fig. 1b. The air bladder can be any shape but is sized to fit easily within chamber 22 when deflated.

Adjacent the air bladder 22 is a rigid plastic occlusion member 24 which is extendible from the housing. The air bladder may be fully retracted within the housing 21, as shown in Fig. 1a. As the air bladder 23 is inflated the occlusion member 24 is forced to extend out of the opening 25 in the inner face of the housing, as shown in Fig. 1b.

The occlusion member 24 includes ledges 241 and 242 which are brought into abutment with the internal wall of housing 21 when air bladder 22 reaches a certain degree of inflation. This position defines the maximum possible extension of the occlusion member from the housing. Occlusion member 24 includes contact surface 243 which lies flush with or below the housing inner face 211 when the air bladder is deflated. As the air bladder is inflated the plane of contact surface 243 extends beyond the plane of inner face 211. In the embodiment shown in Fig. 1 the contact surface 243 is rectangular in shape.

Body 2 also includes lid 26 which covers a rectangular opening 27 in the housing. Opening 27 provides access to chamber 22 so that the air bladder and/or occlusion member can be inspected and replaced if necessary.

The housing 21 is intended to be placed against a limb 3 of a subject. Within the limb passes an artery 31, shown in cross section in Fig. 1. This could be any artery, but the femoral artery within the upper leg is shown in Fig. 1. When the air bladder is deflated and the occlusion member is in the retracted position, the artery is unaffected and blood continues to flow unimpeded. As the air bladder is inflated and the occlusion member extends from the housing, contact surface 243 presses into limb 3 until artery 31 is occluded and blood flow through the artery is reduced or ceases.

Therefore, the device shown in Fig. 1 is adjustable between two states, the first state achieving arterial occlusion and ischaemia, and a second state providing reperfusion.

The device may be secured against the limb with suitable attachment means such as a strap, belt or clamp (not shown).

The air bladder is connected to an air supply (not shown). The air supply controls the inflation and deflation of the bladder.

Fig. 2 shows two different perspective views of a device 4 according to the present invention, designed using CAD software. The device 4 includes two elongate bodies 4a

and 4b connected at hinge 49. Each body includes housing 41a,41b which defines a chamber 42b (chamber 42a not shown).

Fig. 2a shows outer faces 412a,412b of the housing. Occlusion member 44b is visible within chamber 42b.

Bodies 4a and 4b each include lids 46a and 46b (lid 46b not shown) which cover openings 47a,47b in the housings.

Body 4a is shown with lid 46a in place, with opening 47a not visible. Body 4b is shown with lid 46b removed and opening 47b visible.

Fig. 2b shows inner faces 441a,441b of the housing. Occlusion members 44a, 44b are shown in retracted positions with their contact surfaces 443a,443b lying flush with the inner faces of the housings.

Channels 48a,48b at the ends of the elongate bodies distal from hinge 49 are intended to hold attachment means (not shown) such as a belt or strap. A securing means (not shown) may then pass through bores 481a,481b (shown in both Fig. 2a and 2b) and through the attachment means to secure the attachment means to the body of the device. A second securing means at hinge 49 will secure another part of the attachment means to the body. Where the attachment means is a belt or strap, this creates a loop which may be passed around a limb of the subject and tightened to secure inner faces 441a,441b against the limb.

Lids 46a,46b are secured in place over openings 47a, 47b by passing fixing means such as a screw or bolt through the lids and into recesses 482-487.

The device is pivoted about hinge 49 to provide the correct angle between the two elongate bodies, depending on the anatomy of the particular subject on whom the device is being used. The elongate bodies are intended to be placed along respective inguinal canals of the subject, with inner faces of the housings and contact surfaces of the occlusion members in contact with the limbs.

Fig. 3 shows a prototype device according to the present invention, manufactured based on the design of Fig. 2.

Device 6 is shown in two different orientations in Fig. 3a and Fig. 3b in the same way as Fig. 2. The prototype was manufactured from nylon using Selective Laser Sintering 3D printing techniques. A hook-and-loop fastening strap 61 is made up of two separate strap portions 611 and 612. Strap portion 611 is attached to the body of the device at a point distal from the hinge, and strap portion 612 is attached to the body of the device at the hinge. Gas supply tubes 62a and 62b connect an air bladder (not shown) within the device housing to a gas supply system (not shown).

Fig. 4 shows a combined gas supply and control system 7 for control of the inflation and deflation of the air bladder within the device housing. Nitrogen gas cylinder 71

pressurises gas line 72 with high pressure N₂ gas. Gas supply pressure sensor 731 detects the gas pressure immediately downstream of cylinder 71. Inlet valve 741 can be opened to supply gas to the air bladders within device housing 75a, 75b. Air bladder pressure sensor 732 detects the gas pressure downstream of inlet valve 741 and therefore detects the pressure of gas supplied to the air bladder.

Exhaust valve 742 can be opened to release gas from the system and decrease the pressure downstream of inlet valve 741, thereby deflating the air bladder.

Air reservoir 76 provides additional volume to the gas supply system. This simplifies the fine-tuning of pressure within the system, reducing the risk of over-inflation or rupture of the air bladder.

Microcontroller 77 is connected to inlet valve 741, exhaust valve 742, gas supply pressure sensor 731 and air bladder pressure sensor 732. The microcontroller can therefore completely control inflation and deflation of the device by adjustment of the valves based on readings taken from the pressure sensors.

A typical adjustment procedure during an ischaemia period may occur as follows.

1. Inlet valve 741 and exhaust valve 742 are closed;
2. Inlet valve 741 is opened to pressurise the gas line downstream of the inlet valve and begin air bladder inflation;
3. Inlet valve 741 is closed when the pressure detected by air bladder pressure sensor 732 reaches a predetermined threshold;
4. If the pressure at the air bladder pressure sensor falls below a given threshold, inlet valve is opened until normal pressure is achieved;
5. If the pressure at the air bladder pressure sensor exceeds a given threshold, exhaust valve 742 is opened until normal pressure is achieved.

Microcontroller 77 controls all of these steps, providing an automated system.

Examples

Methods

The following Examples relate to rapid remote ischemic postconditioning following global hypoxic ischemic insult in a piglet model of perinatal asphyxia. The treatment effect was explored using clinically relevant biomarkers of in vivo (¹H) magnetic resonance spectroscopy (MRS) for lactate, N-acetyl aspartate, creatine (Cheong et al., 2006, Thayyil et al., 2010) and ³¹P-MRS for inorganic phosphate, phosphocreatine and nucleotide triphosphate (NTP) (Azzopardi et al., 1989). Cell death and microglial activation was quantified at 48 hours post insult using transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL), cleaved caspase 3-positive cells and microglial ionized calcium-binding adaptor molecule 1 (IBA1) antibody staining for immunohistochemistry. To assess underlying neuroprotective mechanisms, western blots and microarray tests were performed on cortical and corpus callosum.

Animal experiments and surgical preparation

Table 1 shows statistics for the control and RIPostC sample groups. There was no significant difference in age, weight and insult severity between two groups.

Table 1

Mean (SD)	Controls	RIPostC
Age (h)	35.8 (10.9)	33.0 (10.0)
Body Weight (kg)	1.80 (0.22)	1.83 (0.21)
Duration of HI (min)	20.5 (2.6)	20.6 (1.9)
Insult Severity ($\times 10^{-2}$ min)	9.6 (3.3)	9.1 (3.10)

All animal experiments were performed under aseptic condition and UK Home Office Guidelines [Animals (Scientific procedures) Act, 1986]. Sixteen female piglets, aged less than 30 hours, with a weight range of 1.6-2.1 kg were anaesthetized and surgically prepared as described elsewhere (Lorek et al., 1994). Following initial assessment for any signs of obvious infection including diarrhea and conjunctivitis, an intramuscular dose of 0.2 mg/kg of midazolam was given into the buttock for sedation. Anaesthesia was induced by 4% v/v isoflurane given through a facemask for few minutes to facilitate tracheostomy and intubation. Throughout the surgery isoflurane was maintained at 2.8-3% guided by peripheral oxygen saturation monitoring (Nonin Medical, Plymouth, MN, USA) and the animal's response to stimulation. Following tracheostomy, a suitable size of endotracheal tube (Smiths Medical, Ashford, Kent, UK) was fixed and the piglet was mechanically ventilated (SLE 2000 infant ventilator, Surry, UK). Ventilator settings were adjusted following measurement of arterial blood gas to maintain partial pressure of oxygen (PaO_2) at 8–13kPa and carbon dioxide (PaCO_2) at 4.5–6.5kPa, allowing for temperature and fraction of inspired oxygen (FiO_2) correction of the arterial blood sample. After the airway was secured, both common carotid arteries (CCA) were surgically isolated at the level of the fourth cervical vertebra and a vascular occluder (OC2A, In Vivo Metric, Healdsburg, CA, USA) was placed on each side. After completion of surgery, inspired isoflurane concentration was maintained at 2% v/v.

An umbilical venous catheter (UVC) was inserted for infusion of maintenance fluids (10% dextrose, 60 ml/kg/day before the insult and 40 ml/kg/day after resuscitation), fentanyl (5 $\mu\text{g/kg/h}$), and antibiotics (benzyl penicillin 50 mg/kg, every 12 hours and gentamicin 4 mg/kg, once a day). An umbilical arterial catheter (UAC) was inserted for invasive physiologic monitoring (SA instruments) for heart rate and arterial blood pressure, and necessary blood sampling including blood gas and electrolytes (Abbot Laboratories, UK). Hepsal (0.5 IU/ml of heparin in 0.9% saline solution) was infused at rate of 0.3 ml/hr to prevent UAC blockage.

After surgery, and before securing piglets in the plastic pod, the remote ischaemic postconditioning device was placed over the inguinal canals and strapped securely in a cross shape with an inflatable bladder under the cross for further fixation. Fig. 7 shows how the device was attached.

To assess the right hind limb blood perfusion during and after ischemic postconditioning, a separate pulse oximeter was attached to right hind limb; while laser Doppler assessed perfusion on the left side. An additional pulse oximeter was attached to right forelimb to monitor the systemic oxygen saturation. The efficacy of ischemia was confirmed by the loss of oxygen saturation in left hind limb and limb blood flow by laser Doppler in right hind limb. Fig. 4 shows some laser Doppler traces obtained during the ischaemia cycles. The top trace in each plot shows the occluder pressure while the bottom trace demonstrates blood flow traced by laser Doppler. Upon increasing the pressure in the occluder, limb blood flow reduces and finally stops. Blood flow reestablishes upon deflation of the occluder.

The piglets were cared for under intensive condition throughout the experiment. To maintain the mean arterial blood pressure (MABP) above 40 mmHg, bolus infusions of 0.9% saline (Baxter; 10ml/kg), dopamine (5-20 μ g/kg/min), dobutamine (5-20 μ g/kg/min) and adrenalin (0.1-1.5 μ g/kg/min) were used as required. High serum lactate was managed by optimizing the oxygenation and half saline bolus. Hyperkalemia ($K > 7.0$ mmol/l) was managed by 4 mcg/kg of salbutamol via UVC over 10 minutes. Salbutamol was diluted to 10 mcg/ml and was repeated every 1- 2 hour if needed. Additionally, 0.5 ml/kg of 10% calcium gluconate was slowly given to stabilise the myocardium when ECG changes or hypocalcaemia (ionized calcium < 1 mmol/l) was present. In the presence of metabolic acidosis, half correction of sodium bicarbonate was given only after ensuring adequate ventilation.

To facilitate the acquisition of magnetic resonance spectroscopy (MRS) data, piglets were positioned prone within a plastic pod. The head was immobilised in a stereotactic frame which included near infra-red spectroscopy (NIRS) optodes placed against the sides of the head.

MR Methods

Piglets were positioned within the bore of 9.4 Tesla Agilent MR scanner (Fig. 17). ^1H and ^{31}P magnetic resonance spectroscopy (MRS) were acquired at baseline and at 24 hrs and 48 hrs after cerebral HI.

^{31}P MRS

A 7cm x 5cm elliptical transmit-receive MRS surface coil tuned to the ^{31}P resonant frequency (51.6 MHz) was positioned on top of the head. ^{31}P MRS was acquired with 1 min resolution using a non-localised single-pulse surface-coil acquisition (repetition time 10 s, 6 summed acquisitions per spectrum). MRS data were analysed using AMARES (Vanhamme et al., 1997) as implemented in the jMRUI software. Prior knowledge of NTP multiplet structure was used (fitting doublets to α - and γ -NTP and a triplet to β -NTP) but no assumption was made as to multiplet relative sizes. NTP is predominately ATP and the latter contributes approximately 70% of the NTP signal e.g. in the rat pup (Mandel and Edel-Harth, 1966). Thus NTP changes during this experiment predominately reflected ATP changes. Pi was fitted using 4 separate components and PCr with a single component. The following peak-area ratios were calculated: Pi/epp, PCr/epp, and NTP/epp where epp = exchangeable phosphate pool = Pi + PCr + 2 γ -NTP + β -NTP.

¹H MRS

¹H MRS data were collected from voxels located in the dorsal right subcortical white matter at the centrum semiovale level (WM voxel, 8x8x15mm) and in the deep grey matter centred on both lateral thalami (DGM voxel, 15x15x10mm) using a combination of a 65 x 55 mm elliptical receive surface coil, a 150 mm transmit volume coil and a LASER acquisition (TR = 5000ms, TE = 288 ms, 128 averages). Spectra were analysed using AMARES as implemented in the jMRUI software and the Lactate (Lac) / N-acetyl aspartate (NAA) peak area ratio was calculated.

Cerebral hypoxia–ischaemia (HI)

NIRS and ³¹P MRS were acquired continuously for 10 minutes at baseline, during HI and for 1 hour after cessation of HI. HI was induced inside the MR scanner by remotely inflating the vascular occluders around both common-carotid arteries, and simultaneously reducing fractional inspired (Fi) O₂ to 6% (vol/vol). During HI the β-NTP peak height was continuously monitored using in-house Matlab (Mathworks) software. As shown in Fig. 5, at the point at which β-NTP had fallen to 50% of its height at baseline, Fi O₂ was increased to 9%. At the point at which β-NTP had fallen to 40% of its height at baseline the inspired oxygen fraction was then titrated to interactively keep the β-NTP peak height between 30% and 40% of its original height for a period of 12.5 min. At the end of HI the carotid arteries were de-occluded and the inspired oxygen fraction returned to 21%. Insult severity was estimated by calculating the time integral of the change in NTP/epp during HI and the first 60 min of resuscitation, as described previously (Faulkner et al., 2011).

Remote ischemic postconditioning method/device

An external IPostC device was designed using CAD software (Autodesk Inventor Professional 2013) and then manufactured using a 3D printing process (Selective Laser Sintering) from nylon. A “V” shaped device with protruding parts in each arm was secured by Velcro straps in a position, which was directly over the femoral artery. The occlusion of the femoral artery was induced by an outward thrust of a rectangular structure following remote inflation of bladders behind the protruding structures (see Fig. 1). A control system (Fig. 4) was also built to provide consistent inflation pressure during the ischaemia cycles, and ensure that the air bladders remained deflated at all other times. This controller consisted of two solenoid valves (2 port VDW10 series, SMC Pneumatics LTD, Crownhill, UK), one to gate the inflow of high pressure nitrogen for inflation, and the second as an exhaust to control deflation. Air bladder pressure was measured using an electronic sensor (MPX5050, Freescale Semiconductor, Arizona, US) and monitored continuously using a microcontroller (Arduino Uno), providing feedback for when to inflate and deflate. During inflation each bladder was maintained at a pressure of at least 40kPa. An LCD display was used to provide visual feedback of the bladder pressure, and display the seconds elapsed since the last inflation/deflation. In addition an analogue voltage proportional to the pressure of the air bladder within the device, and a digital gating signal (high when inflated) were provided at BNC sockets on the front panel for independent monitoring and recording.

Experimental groups

As shown in Fig 6, following resuscitation, while still in the bore of magnet piglets were randomized to either control (HI) or remote ischemic postconditioning (RIPostC). There were 8 animals in each group. In the control group, piglets had only the hypoxic ischemic insult without inflation of postconditioning device bladder. In the RIPostC group, immediately after resuscitation the piglets underwent 4 cycles of 10 minutes ischemia in both hind limbs followed by 10 minutes reperfusion by remotely controlled inflation and deflation of the device bladder. Limb ischemia was ascertained by asphygmia and sphygmus upon inflation and deflation of the postconditioning device in the right hind limb and laser Doppler velocimetry in the left hind limb. Both groups were looked after intensively for 48 hours and were kept normothermic (38-38.5°C).

Electroencephalography and amplitude-integrated electroencephalography

Six lead EEG was acquired throughout the experiment when the piglets were outside the bore of the magnet. Filtered amplitude integrated EEG (aEEG) was then classified based on their voltage pattern similar to Toby study (www.npeu.ox.ac.uk/TOBY). Grade 3 is given to normal voltage (upper margin $>10\mu\text{V}$, lower margin $>5\mu\text{V}$). Grade 2 is moderately abnormal voltage (upper margin $>10\mu\text{V}$, lower margin $\leq 5\mu\text{V}$). Grade 1 is severely abnormal (upper margin $<10\mu\text{V}$, lower margin $<5\mu\text{V}$). The grading was done at baseline (before insult), 3, 6, 12, 24, 36 and 48 hours post insult.

Additionally to detect more accurately any difference between groups, the EEG power trend was assessed in both groups. EEG data for inter hemispheric channel C4-C3 was segmented into 2-minute epochs and fast Fourier transform (FFT) was performed with a Hanning window using the Nicolet One power trend software for total power (0.5-30Hz) for all EEG recorded.

Median hourly power values were taken for each hour in order to avoid any artifacts caused by handling. Power values were worked out from 3-48 hours. Baseline power value was considered as 100 percent and then each median power value was calculated relative to the baseline (100%). The reasons for this percentage conversion was to eliminate any inter subject variability. The area under the curve (AUC) for both median values and percentages for 3-48 hours after the insult was calculated in order to evaluate the recovery. EEG power recovery was calculated for each subject as the area under the curve (AUC) for percentage of baseline power for 3-48 hours.

Near Infrared Spectroscopy (NIRS)

Near Infra-Red Spectroscopy (NIRS) data was acquired at baseline, during the insult and after resuscitation and throughout the remote postconditioning cycles. An "in house" constructed broadband spectrometer previously used in piglets and the human brain was used (Springett et al., 2000, Tisdall et al., 2007).

Changes (Δ) in the brain concentrations of oxygenated haemoglobin (HbO_2), deoxygenated haemoglobin (HHb), and oxidized Cytochrome c oxidase (CCO) were determined using the UCLn algorithm (Matcher et al., 1995) after correction for the wavelength dependence of path-length (Essenpreis et al., 1993). Changes in haemoglobin difference ($\Delta[\text{Hbdiff}] = \Delta[\text{HbO}_2] - \Delta[\text{HHb}]$; indexing changes in brain

oxygenation) and changes in total haemoglobin ($\Delta[\text{Hb}_{\text{tot}}] = \Delta[\text{HbO}_2] + \Delta[\text{HHb}]$; indexing cerebral blood volume) were also calculated (see Fig. 10).

Mean data were compared between RPostC and control by using Wilcoxon-rank test and $p < 0.05$ was considered as significant.

Brain histology

Piglets were euthanised by pentobarbital injected via UVC at 48 hours after insult. The brain was fixed through cardiac perfusion with cold 4% paraformaldehyde in PBS. After seven days fixation in 2% paraformaldehyde the brain was dissected. Five-millimeter thick coronal slices of the right hemisphere from optic chiasma were embedded in paraffin wax and sectioned and stained for haematoxylin and eosin. Right hemisphere samples were used for immunohistochemistry and histology studies. Fresh brain samples from the left hemisphere were used for western blot and microarray studies. To assess cell death, apoptosis and microglial activation, brain samples were stained for nuclear DNA fragmentation, appearance of activated caspase 3 and microglial ionized calcium binding adaptor molecule 1 (IBA1). An investigator blind to the treatment group performed all the analysis. For each animal 2 sections were stained and 11 different regions in the brain were examined at 2 -3 fields of vision. TUNEL positive nuclei were counted at x40 magnification while caspase 3 and IBA1 counts were at x20 magnifications. $P < 0.05$ was considered as significant.

For all histochemical and immunohistochemical stains, brain sections were dehydrated in xylene (3 x 10 min) and rehydrated in graded ethanol solutions (100–70%), followed by double-distilled water. For TUNEL, the sections were pretreated for 15 min in 3% H_2O_2 in methanol to remove endogenous peroxidase, followed by a 15-min peptidase predigestion with 20 mg/ml proteinase K (Promega) at 65°C, and then incubated at 37°C for 2 hours with the TUNEL solution (Roche) containing biotinylated dUTP. For immunohistochemistry, the sections were processed for antigen retrieval (800-mW microwave irradiation in 0.1 M citrate buffer, 10 min), followed by overnight incubation with primary rabbit antibody against activated caspase 3 (1:500) (Abcam) or IBA1 (1:1000) (Wako), and then 2-h incubation with a biotinylated secondary goat anti-rabbit immunoglobulin antibody (1:100, Jackson Laboratory). The biotin residues were detected with the avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories) and visualized with diaminobenzidine/ H_2O_2 (Sigma), with CoCl_2 and NiCl_2 included to intensify TUNEL histochemistry. The sections were counterstained with haematoxylin, dehydrated in graded alcohol and xylene and mounted with Depex (VWR), or alternatively, mounted with Vectashield + 4',6-diamidino-2-phenylindole (DAPI) aqueous mounting media (Vector Labs), to facilitate total cell number counts during analysis of Iba1 and activated caspase 3. The less numerous activated caspase 3 immunoreactive cells were counted in two fields of view at x20 magnification, and cell number was adjusted to total DAPI- positive cell number. Activated caspase 3 was counted in the periventricular white matter, caudate nucleus, thalamus and in the parasagittal cortex and midtemporal cortex (insular region) containing the superficial, middle and deep cortical layers. The IBA1 counts were made in the periventricular white matter, caudate nucleus and thalamus at x40 in two fields of view within each region and adjusted to total DAPI- positive cell number.

The threshold for statistical significance was $P < 0.05$. For statistical analysis in individual brain regions, original counts of TUNEL were normalized by $\log(x+1)$ algorithm conversion (Werner et al., 2001), and the differences between the two groups detected using 1-way ANOVA, followed by post hoc Tukey test. Cell number adjusted to total cell density for IBA1 and activated caspase 3 was assessed using a Student t-test. Trend analysis was performed across all seven forebrain regions, again using 1-way ANOVA followed by Tukey test. Statistical significance of the R^2 value in correlation plots was assessed using the F-test.

Sample size

The primary outcome, the change in area under the curve for lactate/ N-acetyl aspartate from baseline to 48h, was used to calculate the sample size. Previous work with our model suggested that the change in lactate/N-acetyl aspartate during 48 h varied between normo- and hypothermic groups by 1.0U, with a standard deviation of 0.75U (both log scale). Assuming similar magnitude of additional effect for melatonin-augmented hypothermia (versus hypothermia alone) and with 5% significance and 80% power, nine subjects would be required in each group.

RNA extraction, amplification and microarray hybridisation

At the time of euthanasia an area of periventricular white matter (corpus callosum) was taken within 20 minutes of death, placed in RNAlater solution (Qiagen, West Sussex, UK), frozen in liquid nitrogen and stored at -80°C until processing. RNA was extracted using the standard protocol for animal tissues supplied with the RNeasy Midi kit (Qiagen, West Sussex, UK). RNA used for microarray was assessed using a Nanodrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, USA) and all samples had a spectral 260/280 ratio of between 2.05-2.13, and a RIN of 9.9-10. For each sample 200ng of total RNA was amplified and labeled using an Ambion WT expression kit (Invitrogen, Life Technologies Ltd, Paisley, UK). Briefly total RNA is converted to cDNA and then linearly amplified to create an antisense cRNA library. This is then converted to single strand sense cDNA, which is fragmented and end-labeled before hybridisation using a Gene Chip WT terminal labeling and controls kit (Affymetrix, California, USA). The amplified targets were hybridised to Gene Chip Porcine Genome Arrays (Affymetrix, California, USA) overnight and scanned using Gene-Chip Scanner 3000 7 G. Data files were extracted from the image files automatically by Gene-Chip Command Software (version 2, Affymetrix, California, USA) and the CEL file format was subsequently used for analysis.

Microarray analysis

Analysis was performed using Genespring GX12 (Agilent, California, USA). Data from the individual microarray chips was first normalized across dataset and summarized using the Robust Multi-array Analysis (RMA) algorithm. Data was filtered to include only those probe sets falling between the 20th and 100th percentile after normalization. Initial analysis with Genespring GX12 probe sets detected a total of 74 gene transcript sets. We identified unknown probe sets using Basic Local Alignment Search Tool and BLAST-Like Alignment Tool (BLAST and BLAT) sequence alignment software against the pre-labeled mouse genome. Statistical analysis of microarray data was performed using a

one-way ANOVA followed by a Mann-Whitney unpaired post-hoc test and a Benjamini-Hochberg FDR multiple testing correction. P-values were calculated asymptotically. Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, California, USA) was used to assign the 74 identified genes to a range of known biological functions and metabolic or signaling pathways.

Quantitative reverse transcription polymerase chain reaction

Fresh brain tissue collected for microarray analysis was used for qualitative reverse transcription polymerase reaction (qRT-PCR). Sample preparations, primer design and PCR protocol were similar to that previously described (Fleiss et al., 2012). Primers were designed specifically using the Sus scrofa Ensembl database. The reference genes 14-3-3 protein zeta/ delta (YWAHZ) and ribosomal protein L4 (Rpl4) were chosen to standardize all quantitative experiments (Favrais et al., 2011). For each duplicate sample, we averaged the calculated specific ratio of the gene of interest/reference gene.

Western blots

Piglets were euthanised at 46 hours. White matter (corpus callosum) and cortex were dissected, frozen immediately in liquid nitrogen and stored at - 70°C. Samples were lysed by Tris buffer with Halt protease and phosphatase inhibitors (Pierce, #78427 phosphatase inhibitor, #78438 protease inhibitor, UK). Homogenization of lysate was completed with sonication.

Lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was gently aspirated and protein concentration was calculated with a plate reader measuring optical density absorbance (FLUOstar Omega, BMG Labtech).

Equal amount of protein samples and Laemmli buffer were mixed and the mixture was boiled at 100°C for 10'. Equal amounts of proteins (30 micrograms) were loaded and separated through 10% polyacrylamide SDS running gel in two separate gels for P-Akt and P- Erk, and T-Akt and T- Erk. Separate nitrocellulose membranes were used for blotting the proteins. One membrane was stained with primary antibodies for P-Akt (Cell Signaling technology, #9101, NEB, UK), P-Erk (Cell Signaling technology, #9271, NEB, UK) and tubulin (Abcam, AB7291, UK) as loading control protein. The second membrane stained with primary antibodies for T-Akt (Cell Signaling technology, #4691, NEB, UK), T-Erk (Cell Signaling technology, #4695, NEB, UK) and for tubulin (Abcam, AB7291, UK) as loading control protein. After overnight incubation on a fridge rocker, membranes were washed and then stained with secondary antibodies (Odyssey anti rabbit and anti mouse fluorescent antibodies, LI-COR Biosciences). Stained membranes agitated for 1 hour and after washing were scanned by Odyssey Imaging Systems (LI-COR).

Statistics

When grouped data are used results are mean (standard deviation). MRS data were analysed using a mixed effects linear regression model with a random subject effect included in the model. This model is able to handle missing data without excluding subjects from the analysis. The individual trajectory for each subject is modeled as varying randomly around the fixed estimated slope (conditional on covariates); each observation within a subject will also vary randomly around its individual trajectory.

Measurement timepoints are referred to as baseline, 24 hours post HI and 48 hours post HI. Fitting an interaction term between the time and the treatment group allows different regression slopes in each time and group combination. Predicted values and the difference between groups are calculated at each time point for each metabolite ratio of interest. Significance was assumed for $p < 0.05$.

Insult severity

There was no significant difference in insult severity between the groups: AED (mean (SD)) was 0.091 (0.031) in RPostC and 0.096 (0.032) in controls ($p = 0.72$).

Example 1

Physiological data

A comparison in temporal measurements of SatO_2 , HR and MBP during postconditioning cycles in control and RPostC group are shown in plots (g), (h) and (i) of Fig. 10 respectively. The control group did not show any significant changes in their physiologic data following resuscitation. However, RPostC group showed significant changes in MBP during ischemic reperfusion cycles. MBP increased during limb ischemia ($p < 0.05$) and went back to baseline level during reperfusion period in RPostC group. No significant difference in oxygen saturation and heart rate during the cycles between the groups.

Example 2

EEG/CFM findings

Cerebral function monitoring (CFM) was assessed and graded at baseline and then 3, 6, 12, 24, 36 and 48 hours after resuscitation. Since all piglets had grade 3 (normal) CFM at baseline, the graph comparing the CFM is started at 3 hours after insult.

Fitting an ordered logistic regression model to the data, and allowing for repeated measurements over time in each pig, the estimated OR comparing the score in the PC group to the score in the control group was 3.32 after adjustment for time of measurement; that is the odds of having a score of 3 versus 1 or 2 are more than 3 times higher in the treated group compared to the controls having adjusted for the time of measurement and similarly the odds of having a score of 3 or 2 versus 1 are more than 3 times higher in the treated group compared to the controls. However, this result is not significant at the 5% level ($p = 0.206$). There was no evidence of difference in the effect of treatment at different time points.

Example 3

MRS data

One subject in the remote IPostC group did not have ^1H MRS because of technical problems. One subject in the control group was terminated prior to 48 h. Predicted values were calculated using the mixed effects model. There were no significant differences between the groups in any measure at baseline. DGM ^1H MRS ratios showed no significant differences between controls and IPostC at any time point. WM Lac/Naa is significantly higher in controls than in IPostC group at 48 hours, as shown in Fig. 9a and 9b ($p = 0.005$). NTP/epp ratio is significantly lower at 48 hours in controls than in IPostC group as shown in Fig. 9c ($p = 0.03$).

Example 4**NIRS data**

Changes (Δ) in the brain concentrations of HbO₂, HHb, and oxidised CCO were determined. Changes in haemoglobin difference ($\Delta[\text{HBdiff}] = \Delta[\text{HbO}_2] - \Delta[\text{HHb}]$; indexing changes in brain oxygenation) and changes in total haemoglobin ($\Delta[\text{HBtot}] = \Delta[\text{HbO}_2] + \Delta[\text{HHb}]$; indexing cerebral blood volume) were also calculated.

CCO was higher in the first ischaemia period, and then is modulated by the blood pressure changes in cycles 2, 3 and 4 (Fig. 11a).

HB_{tot} was higher in the IPC group throughout the recovery period, as shown by Fig. 11b. HB_{diff} was modulated by the blood pressure changes during ischemic cycles (Fig. 11c).

Example 5

Fig. 10 shows that there was significant increase in mean blood pressure during the ischemic cycles compared to control animals (Fig. 10i). Also there is significant increase in cerebral oxygenation and cerebral blood volume in treated group (Fig. 11c and Fig. 11b respectively).

Example 6**Histopathology data**

Fig. 12 shows the mean TUNEL counts in RPostC and control groups in 8 studied regions. There are significant differences between groups in the periventricular white matter (PWM) and internal capsule (IntCap). Fig. 12b shows the effect of postconditioning in PWM, corpus callosum and IntCap areas ($p < 0.0001$ in each). There was significantly reduced TUNEL positive cells in PvWM ($p < 0.01$), internal capsule ($p < 0.01$) and corpus callosum ($p < 0.05$) in RPostC group correlating with 1H MRS data.

Spearman's rank correlation coefficients were calculated for each area. Dependency between repeated measurements on each pig was tackled by fitting a multilevel mixed effects linear regression model on the log-transformed outcomes and including a random pig effect.

Note the effects and their confidence intervals have been exponentiated, so that the effect is a multiplicative one on the untransformed variables. There was an estimated decrease in mean TUNEL in PWM of 74% in the postconditioning group compared to the control group, with values ranging from a 31% decrease to a 90% decrease being consistent with the data at the 95% level.

Fig. 13 shows co-localisation of TUNEL with S100B and Olig2 in white matter. Double labelling of TUNEL with S100B and Olig2 showed that TUNEL positive cells are astrocytes and oligodendrocytes. RPostC group had reduced cell death.

Example 7

Fig. 14 shows mean IBA-1 scores. Significantly reduced activated microglia in corpus callosum ($p = 0.01$) was observed in the RPostC group.

Example 8Microarray dataGene expression changes in response to a remote post-conditioning treatment:

An initial total of 74 probe sets (or entities) were found to be responsive to our remote post-conditioning treatment at the cut off $P < 0.05$. However when a Benjamini-Hochberg FDR multiple testing correction was applied, this number was reduced to zero, suggesting that the analysis could have detected 3-4 genes by chance. Of these 74 genes 63 were down-regulated and 11 were up-regulated. The entire list of 74 genes is illustrated in Table 2 below. The table shows gene expression changes in response to the remote postconditioning treatment. 74 gene transcripts were altered in response to the postconditioning stimulus. 63 genes were downregulated and 13 genes were upregulated. All fold changes are expressed in relation to the control condition. Changes in expression of shaded genes were validated by RT-qPCR.

Table 2

Affymetrix ID	Fold change	Gene name	Abbreviation
15243170	-3.21	cocaine-and amphetamine-regulated transcript	CART
15332951	-2.64	mitochondrial specific tRNA for aspartate	MITO
15308012	-2.64	Unidentifiable transcript	
15207737	-2.47	protein phosphatase 1 regulatory subunit 1B	PPP1R1B
15201579	-2.47	regulator of G-protein signaling 2, 24kDa (RGS2)	RGS2
15289249	-2.35	matrix Gla protein	MGP
15328197	-2.11	regulator of G-protein signaling 8-like	RGS8
15333709	-2.09	UPF0672 protein Chromosome X open reading frame 36 homolog	Cxorf36
		deleted in autism-related protein 1 isoform precursor	
15226506	-2.03	serglycin (proteoglycan)	SRGN
15289109	-2.00	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	ABCC9
15310315	-1.96	long-chain-fatty-acid--CoA ligase ACSBG1-like	Acsbg1
		Acyl-CoA synthetase bubblegum family member 1	mBG1
15323617	-1.96	endothelin receptor type A	EDNRA
15201042	-1.93	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)	GAD2
15286641	-1.92	probable G-protein coupled receptor 88-like	Gpr 88
		striatal specific G-protein coupled receptor	STRG
15288115	-1.92	calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B-like	PDE1B
15293066	-1.90	carboxypeptidase M	CPM
15247924	-1.86	macrophage scavenger receptor 1	MSR1
		CD204	CD204

Affymetrix ID	Fold change	Gene name	Abbreviation
15323206	-1.85	solute carrier family 4, sodium bicarbonate cotransporter, member 4	SLC4A4
15204772	-1.85	translocation associated membrane protein 1	TRAM1L1
15252420	-1.84	striatin interacting protein 2	STRIP2
15300269	-1.83	EGF latrophilin and 7 transmembrane domain containing 1	ELTD1
15293479	-1.82	ATP-binding cassette, sub-family C (CFTR/MRP), member 9 (ABCC9), mRNA.	ABCC9
15275747	-1.81	Unidentifiable transcript	
15192571	-1.80	ectonucleotide pyrophosphatase/phosphodiesterase 1	ENPP1
15324845	-1.77	protein kinase C delta-binding protein	PRKCD
15244800	-1.76	neuron-specific protein family member 2	NSG-2
15334016	-1.76	V-set and immunoglobulin domain containing 4	VSIG4
15293498	-1.70	ATP-binding cassette sub-family C (CFTR/MRP) member 8	ABCC8
15222416	-1.70	dopamine receptor D3	DRD3
15250027	-1.68	cathepsinZ	CTS2
15213927	-1.67	eukaryotic translation initiation factor 4A1	EIF 4Ai
15285807	-1.67	uncharacterized protein C1 orf 51	C1orf51
15202937	-1.66	plexin domain containing 2	PLXDC2
15243427	-1.66	transcription factor COE1	COE1
		early B cell factor 1	EBF-1
		olfactory neuronal transcription factor	OLF1
15274358	-1.65	RNA binding protein, fox-1 homolog (C.elegans)1	RBFOX1
		Ataxin-2-binding protein 1	A2BP1
15218765	-1.64	v-ets erythro blastosisvirus E26 oncogene homolog2 (avian)	ETS2
15196122	-1.63	integrin, alpha11	ITGA11
15283841	-1.63	lymphocyte antigen 96	LY96
15244711	-1.63	CD 180 molecule	CD180
		lymphocyte antigen 64-like antigen	LY64
15337955	-1.63	interleukin 13 receptor, alpha1	IL13RA1
15244446	-1.63	integrin, alpha2 (CD49B, alpha 2 subunit of VLA-2receptor)	ITGA2
15279650	-1.62	regulator of G-protein signaling 5	RGS5
15225513	-1.61	adenosine A2a receptor	ADORA2A
15222322	-1.61	ELL-associated factor 2	EAF2
15324290	-1.61	B cell scaffold protein with ankrin repeats	BANKS 1
15290632	-1.60	decorin	DCN
15208373	-1.59	C/D box U3 snoRNA	U3snoRNA
15321897	-1.59	heparinase	HPSE

Affymetrix ID	Fold change	Gene name	Abbreviation
15192511	-1.59	phosphodiesterase7B	PDE7B
15331349	-1.58	tissue factor pathway inhibitor 2	TFPI2
15241526	-1.57	transcript almost identical to the ephrin receptor A4	EPHA4
15192662	-1.56	laminin, alpha 2	LAMA2
15188495	-1.55	protein kinase C theta	PKC
15283389	-1.54	plasma glutamate carboxypeptidase	PGCP
		NAAG peptidase	
15328560	-1.54	CD34	CD34
15321651	-1.53	proteinphosphatase 3, catalytic subunit, alpha isozyme	PPP3CA
		Calmodulin dependent stimulated protein phosphatase	
		calcineurin	
15289731	-1.52	von Willebrand factor	VWF
15330977	-1.52	v-ets erythro blastosis virus E26 oncogene homolog 1 (avian)	ETS1
		erythroblast transformation specific (ETS), nuclear protein	
15321280	-1.52	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	SPRY1
15239297	-1.52	RNA-binding protein with multiple splicing	RBPMS
15194555	-1.52	unc-13 homolog C(C.elegans)	UNC13C
15208645	-1.51	chemokine (C-Cmotif) ligand14	CCL14
15180033	1.58	transcript that resembles the anthrax toxin receptor	
15314245	1.61	short unidentifiable transcript	
15301562	1.61	short unidentifiable transcript	
15239349	1.61	short unidentifiable transcript	
15239351	1.64	transcript identical to the EGF-like repeat and discoidin 1-like domain	EDIL 1
15266008		developmentally regulated endothelial cells locus 1 promotor	DEL 1
15244889	1.64	Kv channel interacting protein 1	KCNIP1
15286590	1.68	netrin G2	NTNG2
15332217	1.68	tenascin R (restrictin,janusin)	TNR
15301661	1.71	fatty acid 2-hydroxylase	FA2H
15220531	1.87	FEZ family zinc finger 2	FEZF2
15323657	2.08	Hedgehog interacting protein	HHID

Table 3 below summarizes the top 20 genes whose expression was most down-regulated at 48 post hypoxia ischemia following a remote ischemic post-conditioning treatment. All fold changes are expressed in relation to the control group.

Table 3

Fold change	Gene transcript	Abb	Function
-3.21	cocaine-and amphetamine-regulated transcript	CART	neuroprotective
-2.64	Entire mitochondrial genome	MITO	mitochondrial function
-2.47	protein phosphatase 1 regulatory subunit 1B	PPP1R1B	inhibits signal transduction in cAMP pathway
-2.47	regulator of G-protein signalling 2, 24kDa	RGS2	deactivates G protein signalling, activate GDPase
-2.35	matrix Gla protein	MGP	inhibits bone formation
-2.11	regulator of G-protein signalling 8	RGS8	deactivates G protein signalling, activate GDPase
-2.09	UPF0672 protein Chromosome X open reading frame 36 homolog deleted in autism related protein 1	Cxorf36	unclear but involved in development of autism
-2.03	serglycin (proteoglycan)	SRGN	possible mediator of granule-mediated apoptosis
-2.00	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	ABCC9	forms ATP sensitive potassium channels in vascular tissues
-1.96	long-chain-fatty-acid--CoA ligase ACSBG1 Acyl-CoA synthetase bubblegum family member 1	Acsbg1 mBG1	long chain fatty acid synthesis in the endoplasmic reticulum
-1.96	endothelin receptor type A	EDNRA	vasoconstriction, via activation of phosphatidylinositol
-1.93	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)	GAD2	synthesis of GABA
-1.92	probable G-protein coupled receptor 88 striatal specific G-protein coupled receptor	Gpr 88 STRG	orphan receptor unknown ligand
-1.92	calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B	PDE1B	degrades cAMP and cGMP
-1.90	carboxypeptidase M	CPM	controls monocyte to macrophage differentiation controls nitric oxide release in endothelial cells

Fold change	Gene transcript	Abb	Function
-1.86	macrophage scavenger receptor 1 CD204	MSR1 CD204	mediates the endocytosis of low density lipoproteins in macrophages
-1.85	solute carrier family 4, sodium bicarbonate cotransporter, member 4	SLC4A4	transports bicarbonate ions across cellular membranes
-1.85	translocation associated membrane protein 1	TRAM1L1	transports secretory proteins across endoplasmic reticulum
-1.84	striatin interacting protein 2	STRIP2	non genomic activation of endothelial NO synthase by oestrogen receptor alpha
-1.83	EGF latrophilin and 7 transmembrane domain containing 1	ELTD1	endothelial microvasculature, neutrophil migration

Table 4 below relates to the microarray (Corpus Callosum) results, outlining the changes in gene expression at 48 hours.

Table 4

Fold Change	Gene Name	Function	Validated by RT-qPCR
-3.2	CART - Cocaine and amphetamine-regulated transcript	Neuroprotective via ERK pathway. Vasoconstrictive effects via endothelin A receptor	Yes
-2.47	RGS2, RGS8 Regulator of G-protein signalling 2 and 8	G protein coupled receptor - binding site for autacoids (bradykinin, adenosine and endogenous opioids)	Yes
-2.00	ABCC9 - ATP binding cassette, sub family C	Forms ATP sensitive potassium channels in vascular tissue	Yes
-1.96	EDNRA - Endothelin receptor type A	Activated by endothelin 1 and CART - mediates vasoconstriction	Yes

-1.85	SLC44 - Sodium bicarbonate co-transporter	Maintains intracellular pH	Yes
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Fig. 15 shows a significant down regulation in CART (Erk pathway) & ABCC9 (KATP Channels) genes in RPostC group.

The data demonstrates that post conditioning up-regulates the transcription of cocaine and amphetamine regulated transcript (CART), a molecule known to confer neuro-protective effects in rodent ischemia models. Its exact mode of action is unknown, but it is able to induce phosphorylation of NMDA by protein kinase A and C pathways, induces Erk in cell cultures, attenuates the endoplasmic reticulum stress response induced by cerebral ischemia and reperfusion by up-regulating brain derived neurotrophic factor (BDNF), and mediates the neuroprotective effects of oestrogen in ischemia.

The data also demonstrates that the mitochondrial genome is up-regulated following post conditioning which is a paradoxical result that can be interpreted in two different ways. It suggests that the mitochondria are actively dividing, dysfunctional mitochondria are known to divide during secondary energy failure but mitochondria also divide when the energy demands of a cell are increased. Coupled with our MRI results showing an increase in energy metabolism in post conditioned treated animals, our results probably reflect an active increase in the number of mitochondria being produced in post conditioned treated animals.

The data also demonstrates that 48h following post conditioning there is an active suppression of signal transduction in the cAMP/ cGMP, G protein activated pathways.

Long chain fatty acid synthesis in the endoplasmic reticulum is also increased following post conditioning.

The results also suggest that endothelial functioning has been modified by post conditioning, resulting in vasoconstrictive effects (endothelin receptor type A) and a change in function that may be mediated by an effect on endothelial nitric oxide synthase (striatin interacting protein 2, carboxypeptidase M) or its associated downstream signal transduction pathway (calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B, protein phosphatase 1 regulatory subunit 1B, regulator of G-protein signalling 2, 24kDa and regulator of G-protein signalling 8).

Function/ pathway analysis of transcriptional differences induced by post-conditioning treatment

Datasets were analysed by Ingenuity pathways analysis algorithms and database to identify known canonical pathways induced by remote ischemic post-conditioning. However, we were unable to identify any statistically significant pathways induced by this treatment.

Example 9**Western blots**

Brain samples (corpus callosum) was analysed for Akt, p-Akt, ERK and p-ERK protein expression at 48 hours following reperfusion. The results revealed a significant over expression in p-ERK/T-Erk in treatment group compared to control ($p=0.04$). There was no significant difference in Akt and pAkt protein expression.

Fig. 16 shows representative blots for Akt, Erk & Tubulin in corpus callosum (CC) and cortex for both total and phospho-proteins (a, b). Significant difference ($p=0.04$) in P-Erk 44/T-Erk 44 ratio in CC in RIPC group is suggestive of increased Erk phosphorylation in treated group.

A mixed effects linear regression model with a random pig effect was used since repeated measurements led to dependence between observations within each pig. Also a random pig effect rather than a fixed effect was used in order to tackle missed data without excluding the experiments with some missing data. The individual trajectory for each pig can be thought of as varying randomly around the fixed estimated slope (conditional on covariates); each observation within a pig will also vary randomly around its individual trajectory. Fitting an interaction term between the time and the treatment group allows different regression slopes in each time and group combination

Gene expression changes directly related to the efficacy of post-conditioning in other RIPC paradigms

These Examples demonstrate that 48h following hypoxia ischemia and a remote post conditioning treatment there are changes in expression of a number of genes associated with the induction of cardio-protection following a number of other pre and post conditioning paradigms. In particular activation of ATP sensitive potassium channels in vascular tissue is required to facilitate the cardio protective effects of pre and post-conditioning (MCallister et al., 2014), we have demonstrated that a transcript required for the formation of ATP sensitive potassium channels ATP-binding cassette, sub-familyC (CFTR/MRP), member 9 (ABCC9) is down-regulated by -2.00 fold in the microarray analysis and -2.00 fold ($p=0.0222$) when using qRT-PCR. Activation of the endothelin A receptor is also required for pre-conditioning and we have demonstrated that the endothelin receptor A is down-regulated by -1.96 fold in our microarray studies and by -3.00 fold ($p=ns$) using qRT-PCR. Expression of its putative receptor ligand CART is also reduced by -3.21 fold in our microarray analysis and -3.00 fold ($p=0.049$) using qRT-PCR.)

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Claims

1. A device for remote ischemic conditioning including a body configured to attach to a limb of a subject, wherein the body comprises:
 - (i) a housing which provides an inner face for contacting the limb;
 - (ii) a rigid arterial occlusion member which is reversibly extendible beyond the inner face of the housing; and
 - (iii) an occlusion air bladder within the housing, wherein inflation of the occlusion air bladder causes the occlusion member to extend beyond the inner face of the housing.
2. A device according to claim 1, comprising an auxiliary air bladder outside the housing and operable independently from the occlusion air bladder, wherein the auxiliary air bladder is configured to contact the limb such that inflation of the auxiliary air bladder tightens the housing of the device against the limb.
3. A device according to claim 1 or 2, wherein the body is configured to attach to a leg of a subject such that the arterial occlusion member overlies and acts upon the femoral artery to occlude the femoral artery when the occlusion air bladder is inflated.
4. A device according to any one of the preceding claims, wherein the housing defines
 - (i) a chamber containing the occlusion air bladder and at least part of the retracted occlusion member, and
 - (ii) an opening in the inner face through which the occlusion member can be reversibly extended.
5. A device according to claim 4, wherein the occlusion member includes retaining means for retaining at least part of the occlusion member within the housing.
6. A device according to claim 5, wherein the retaining means is a collar of the occlusion member which is larger than the opening and is retained within the chamber when the occlusion air bladder is inflated.
7. A device according to any one of the preceding claims, wherein the body comprises a hatch to provide access to the housing interior.
8. A device according to claim 7, wherein the hatch is a removable panel reversibly attached to an outer face of the housing.
9. A device according to any one of the preceding claims, wherein the body comprises attachment means for securing the device to the limb of the subject.
10. A device according to claim 9, wherein the attachment means is a belt, strap or clamp configured to encircle or grasp the limb.
11. A device according to any one of the preceding claims, wherein the device includes two or more bodies as defined in any one of the preceding claims, each body being configured to attach to a different limb of the subject.

12. A device according to claim 11, wherein the device includes two bodies connected to each other by a hinge and each configured to attach to a different leg of the subject.

13. A device according to any of the preceding claims, further comprising a gas supply system connected to the occlusion air bladder.

14. A device according to claim 13 wherein the gas supply system includes

- (i) a gas source;
- (ii) an inlet valve downstream of the source;
- (iii) an exhaust valve; and
- (iv) a gas reservoir.

15. A device according to any one of the preceding claims, comprising a control system for controlling the inflation of the occlusion air bladder to achieve the required arterial occlusion.

16. A device according to claim 15, wherein the control system includes

- (i) a gas source;
- (ii) a gas supply pressure sensor connected to the gas source;
- (iii) an inlet valve downstream of the gas supply pressure sensor;
- (iv) a device pressure sensor downstream of the inlet valve;
- (v) an exhaust valve;
- (vi) a gas reservoir; and
- (vii) a microcontroller connected to the pressure sensors and valves.

17. A method of performing remote ischaemic conditioning on a subject, including the steps of:

- (a) attaching a device as defined in any one of claims 1 to 16;
- (b) inflating the occlusion air bladder and maintaining inflation at a predetermined pressure for a predetermined period of time to cause the occlusion member to extend beyond the inner face of the housing and apply pressure to the limb, thereby occluding an artery within the limb;
- (c) deflating the occlusion air bladder and maintaining deflation for a predetermined period of time to retract the occlusion member; and
- (d) optionally repeating steps (b) and (c) a predetermined number of times to complete the remote ischaemic conditioning.

18. A method according to claim 17, wherein the remote ischaemic conditioning is performed after the occurrence of an ischaemic event in the subject.

19. A method according to claim 17 or 18, wherein the subject is a neonate and the remote ischaemic conditioning is performed to treat perinatal asphyxia.

20. A method according to claim 17, wherein the remote ischaemic conditioning is performed prior to surgery performed on the subject.

21. A method according to claim 20, wherein the subject is a neonate and the surgery is cardiac surgery.

22. A method according to any one of claims 17 to 21, wherein the device is attached to a leg of the subject and the artery occluded in step (b) is the femoral artery.

23. A device according to any one of claims 1 to 16, adapted for use in a method according to any one of claims 17 to 22.

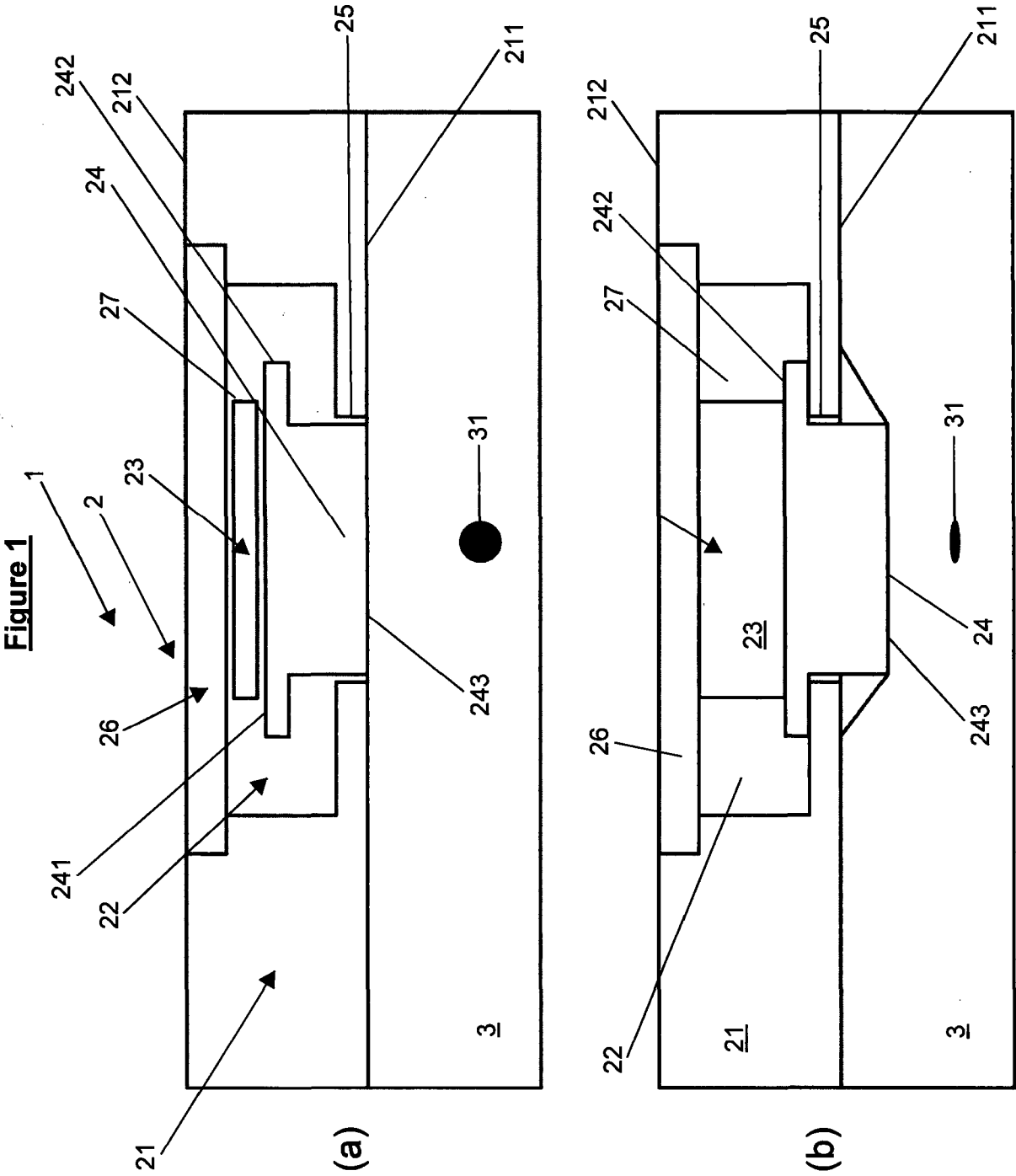
24. A body for use in the device according to any one of claims 1 to 16, configured to attach to a limb of a subject, wherein the body comprises:

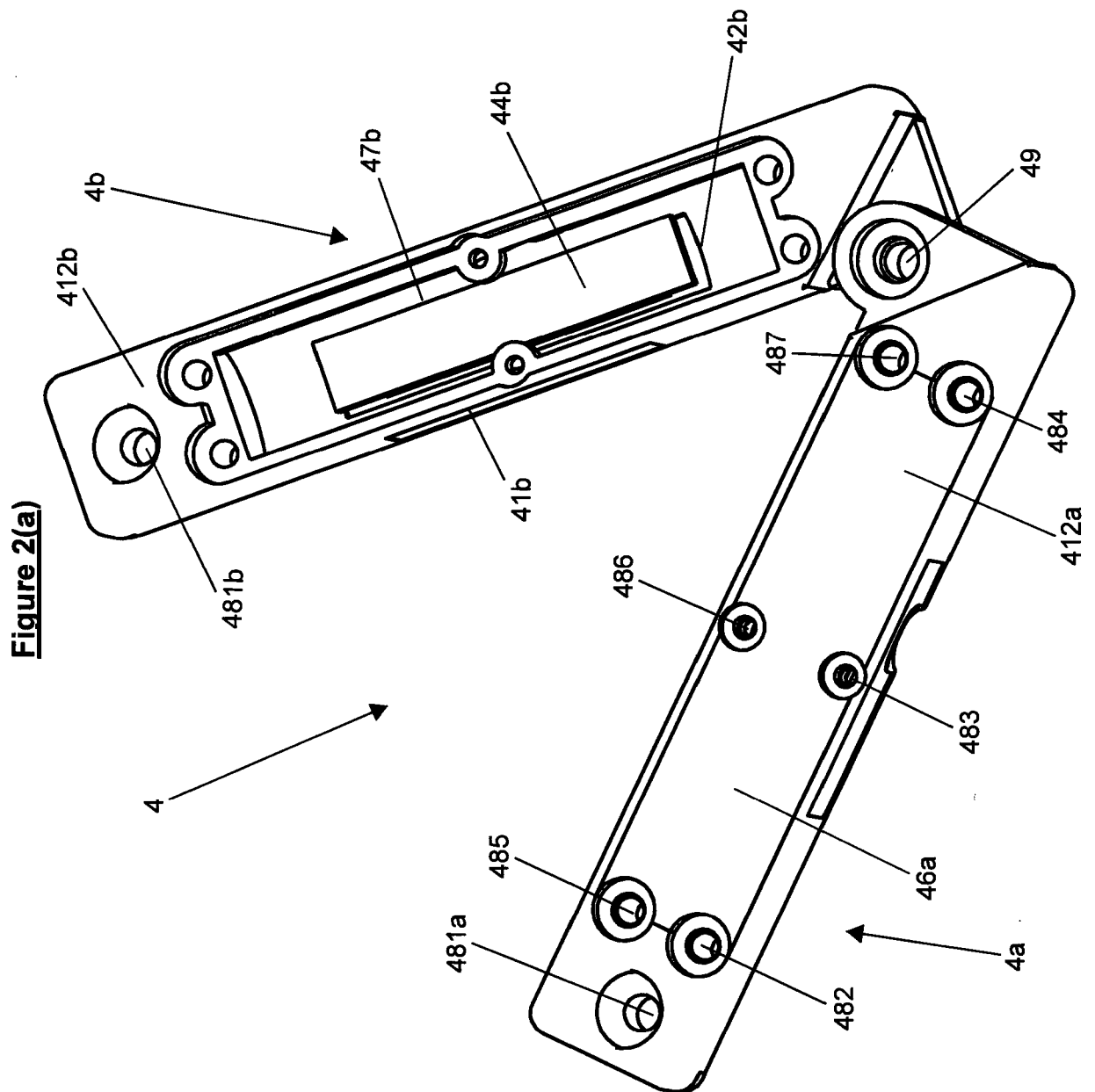
- (i) a housing which provides an inner face for contacting the limb;
- (ii) a rigid arterial occlusion member which is reversibly extendible beyond the inner face of the housing; and
- (iii) an air bladder within the housing, wherein inflation of the bladder causes the occlusion member to extend beyond the inner face of the housing.

25. A device substantially as described in any one embodiment herein, with reference to the drawings.

26. A method substantially as described in any one embodiment herein, with reference to the drawings.

Figure 1





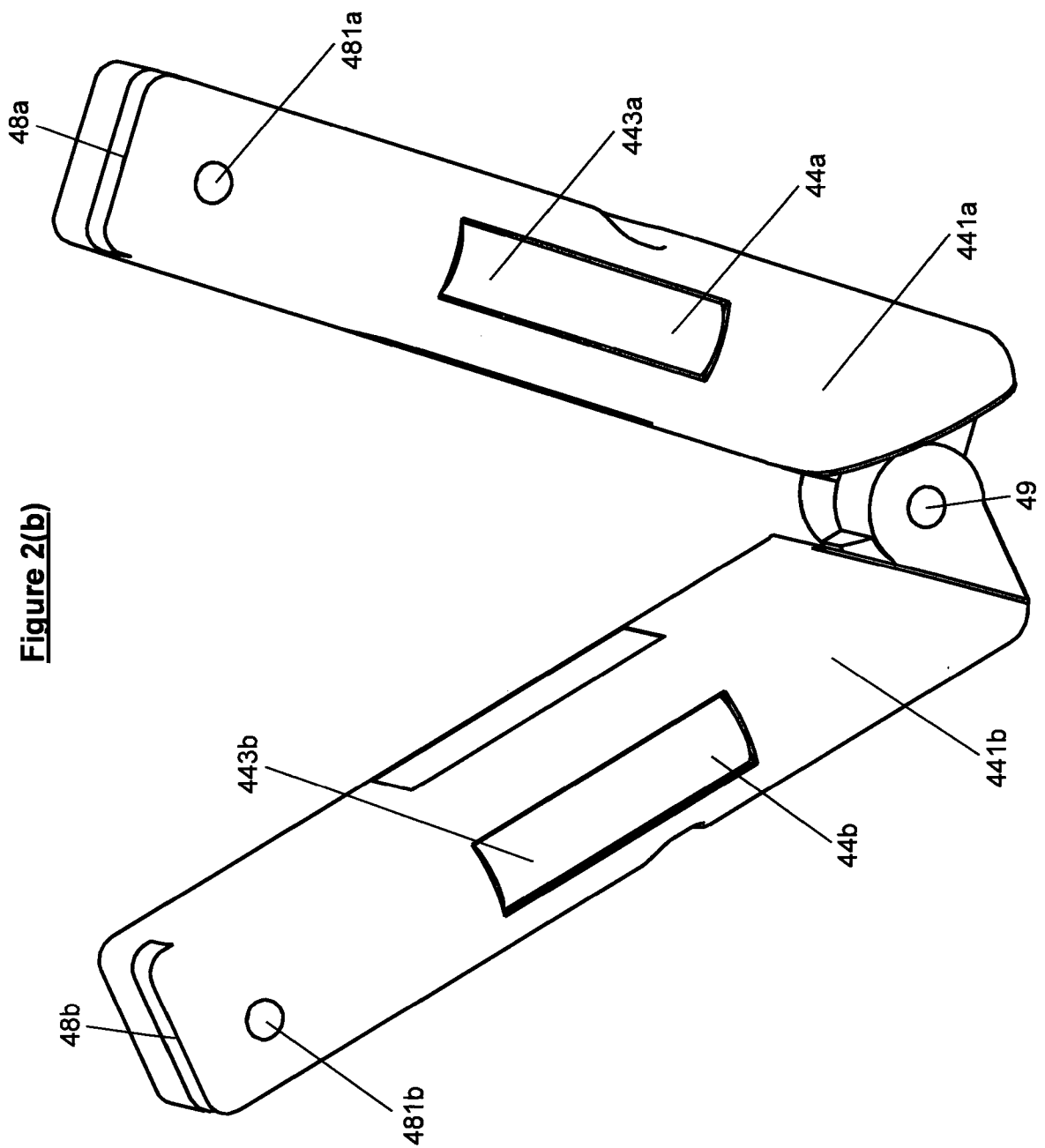


Figure 3(a)

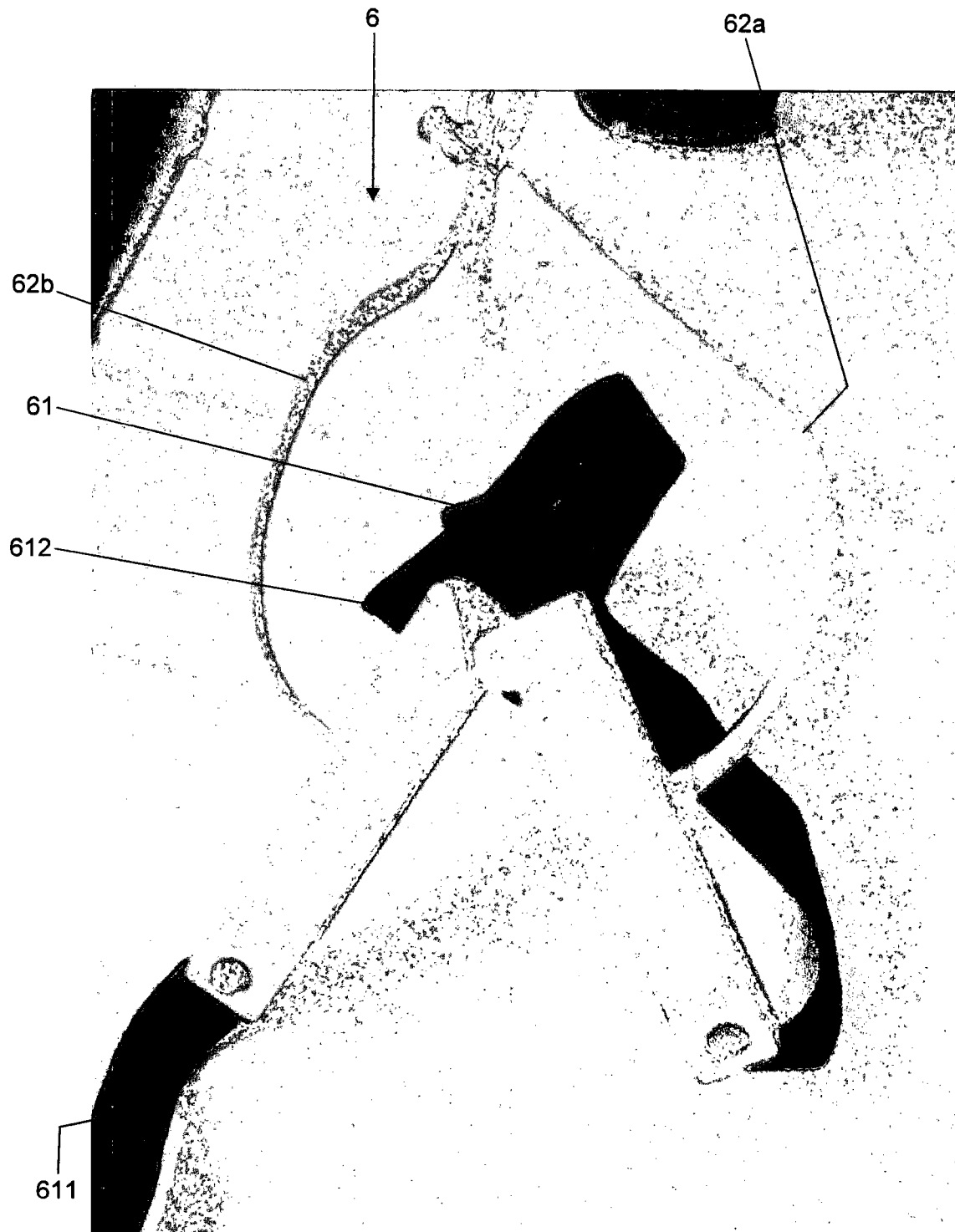
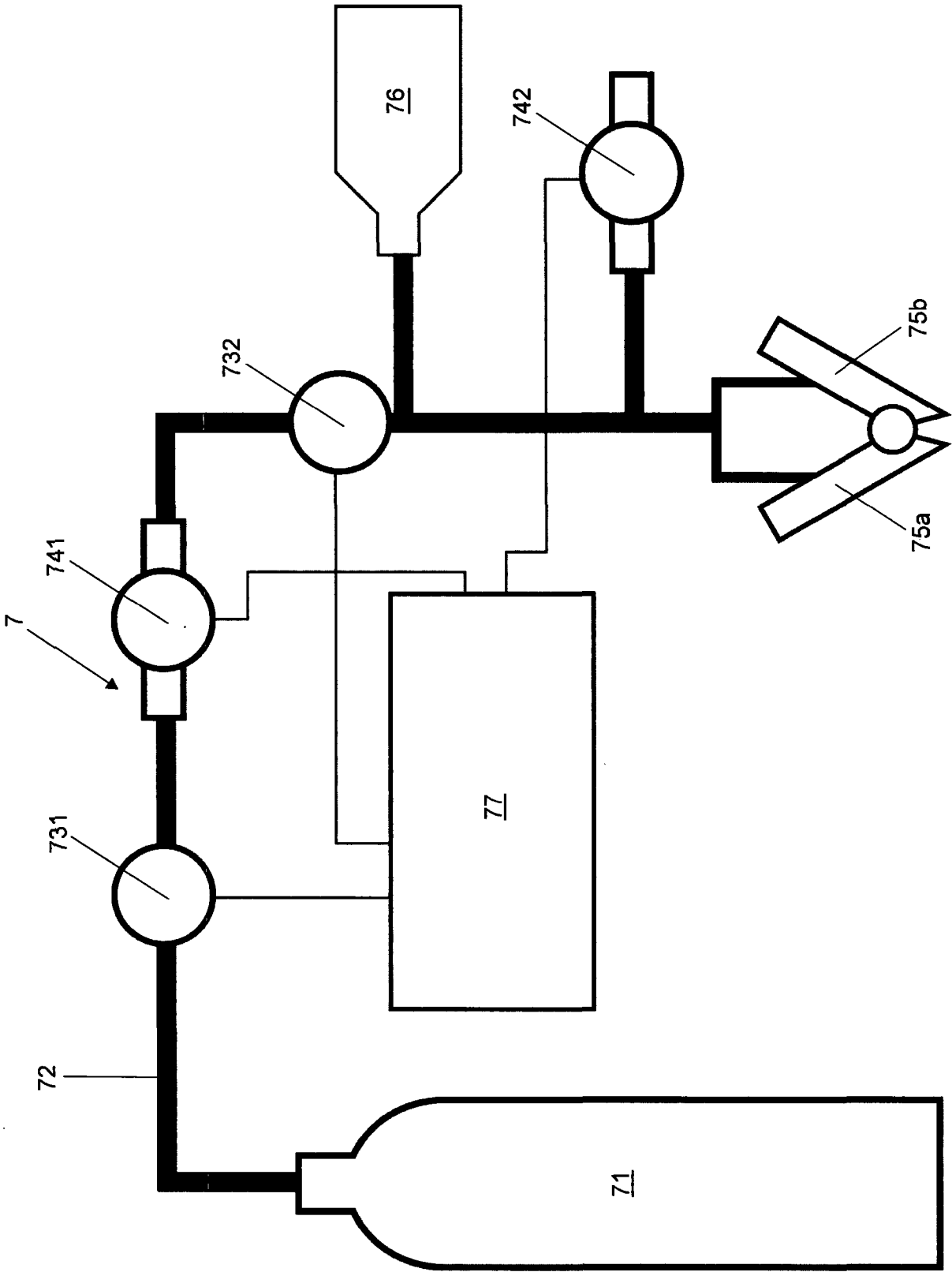


Figure 3(b)



Figure 4



7/18

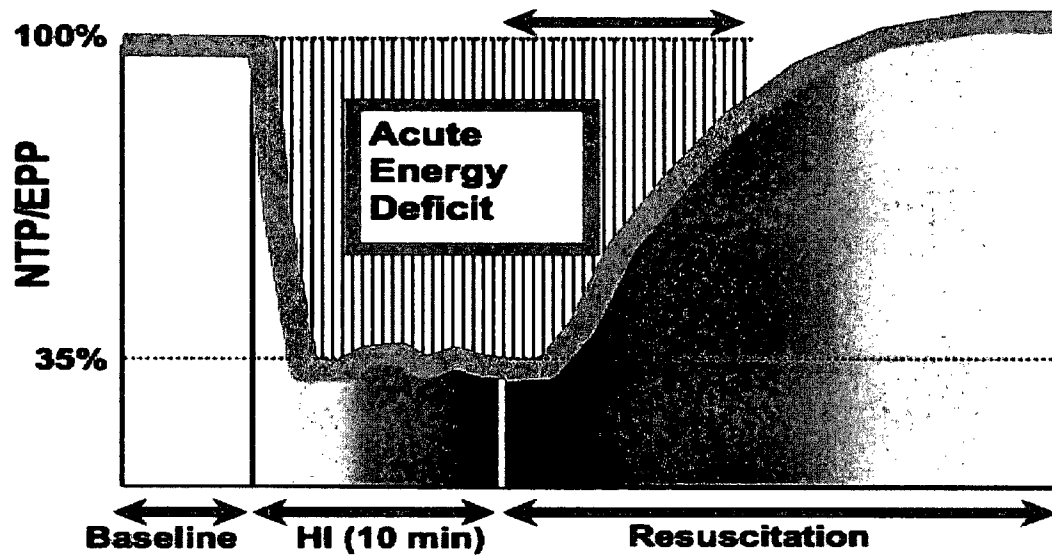


Figure 5

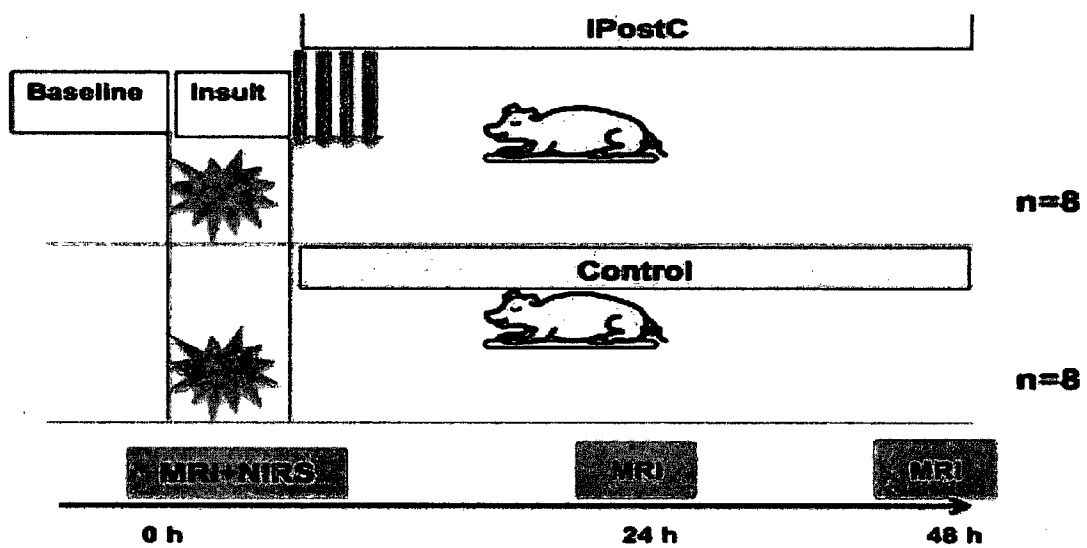


Figure 6

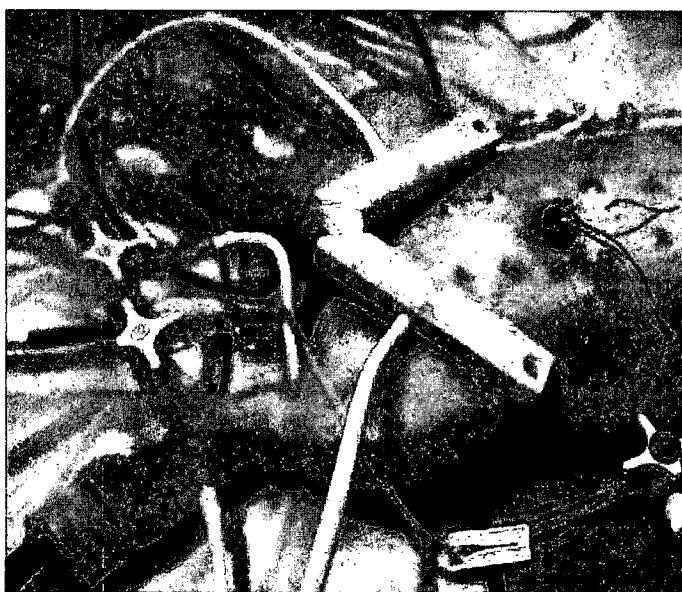
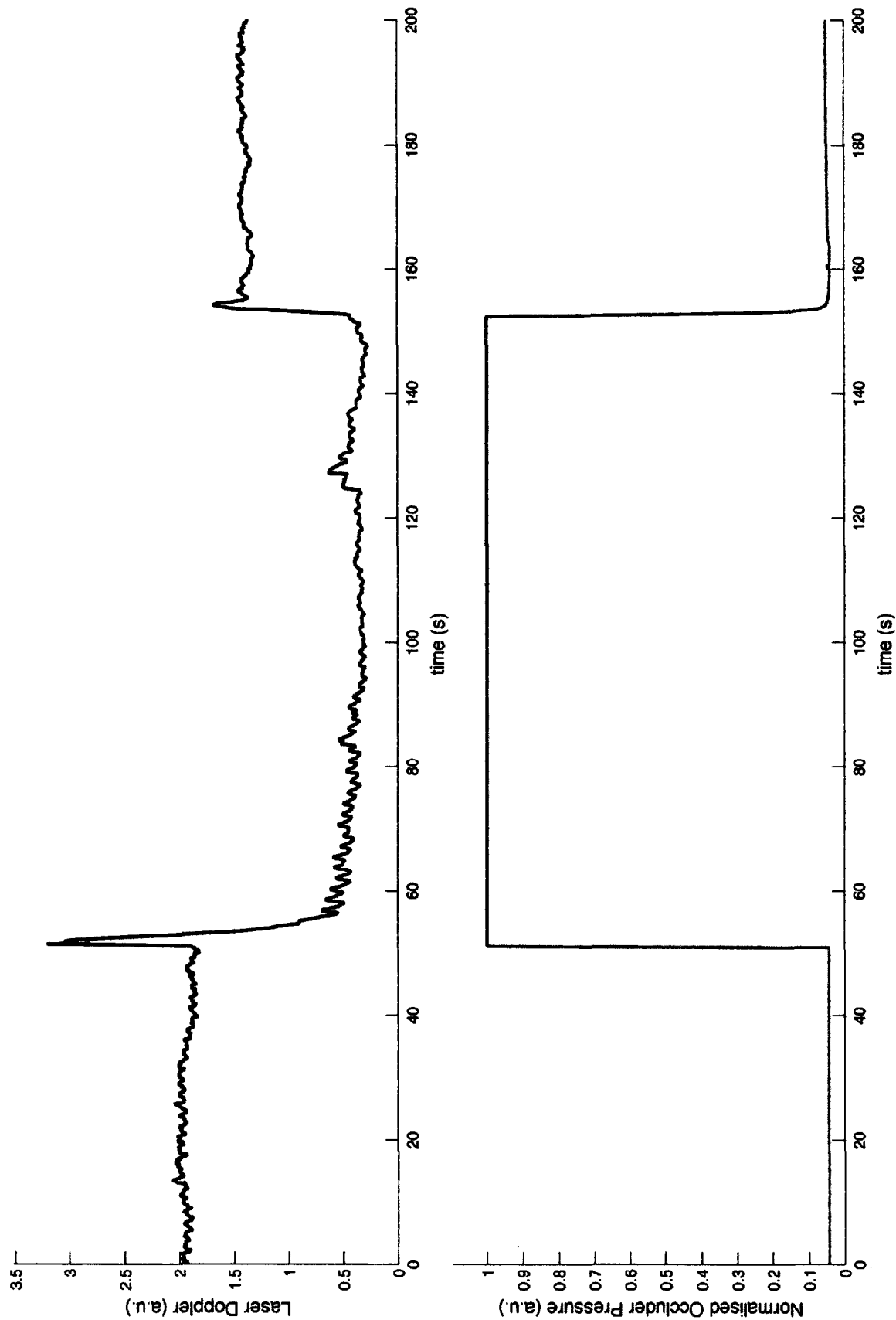
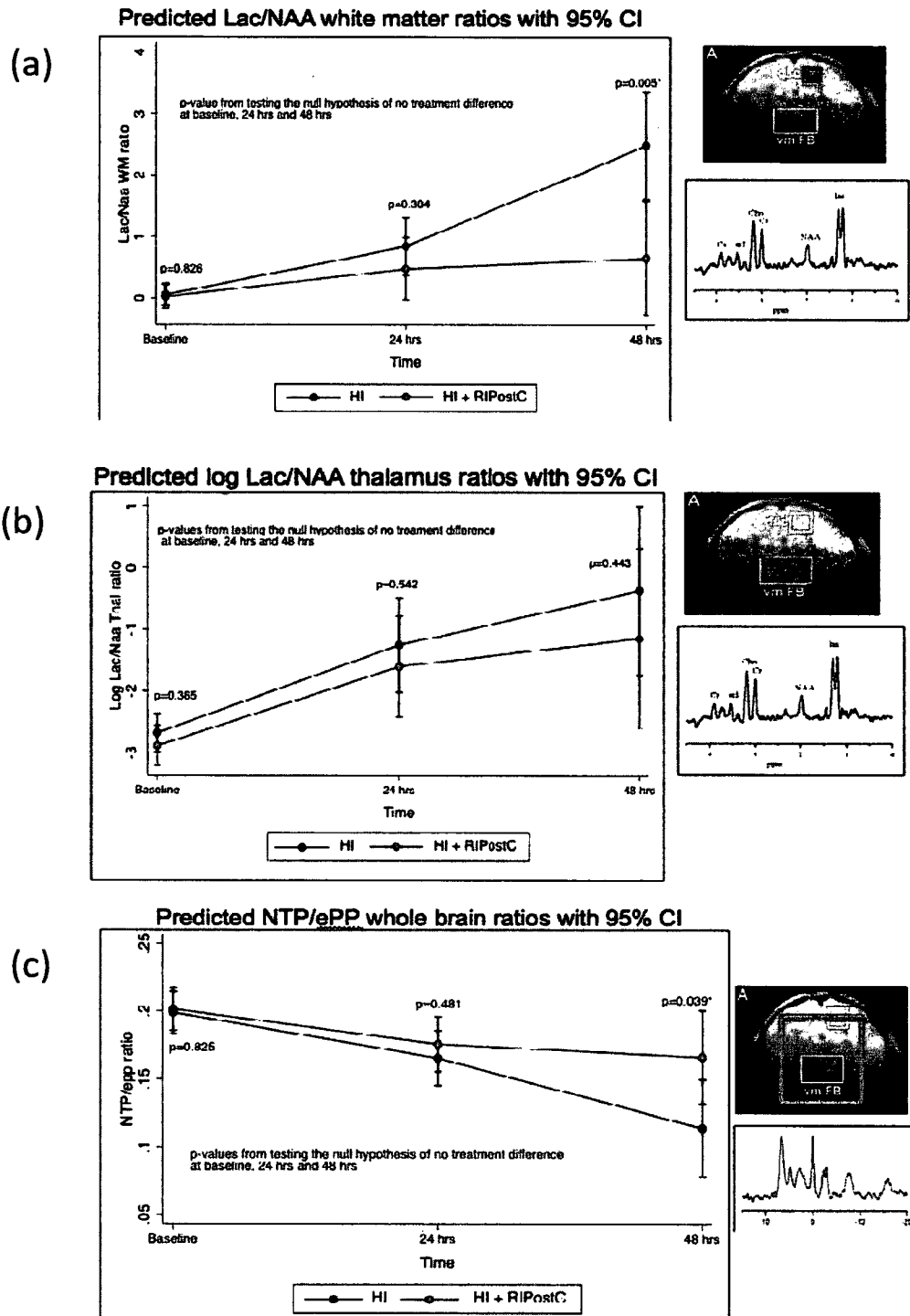


Figure 7

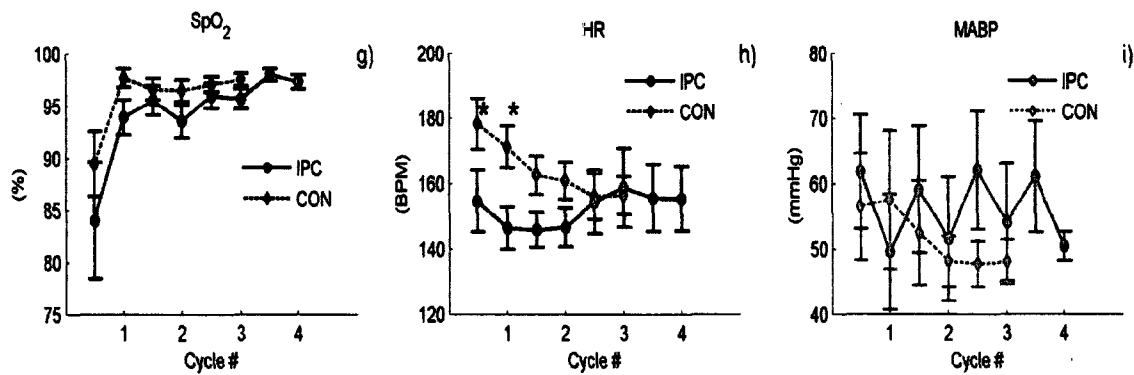
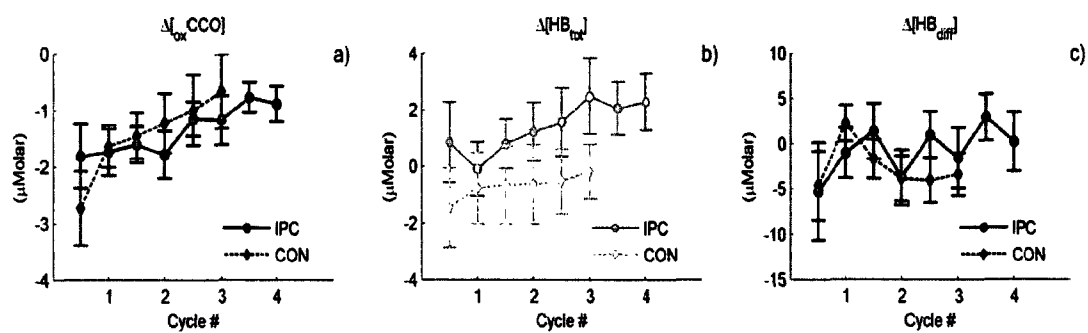
Figure 8



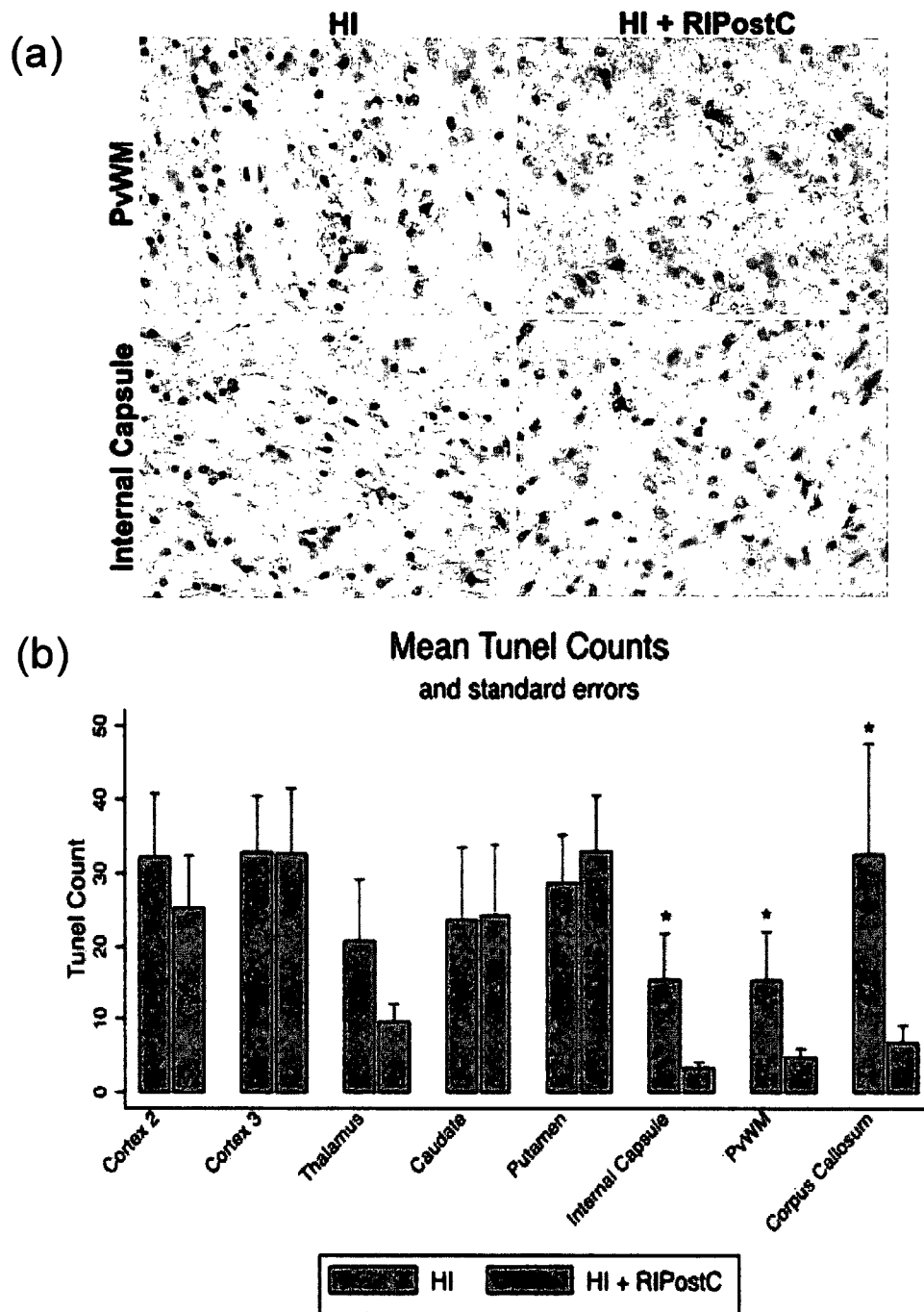
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**Figure 9**

11/18

**Figure 10****Figure 11**

12/18

**Figure 12**

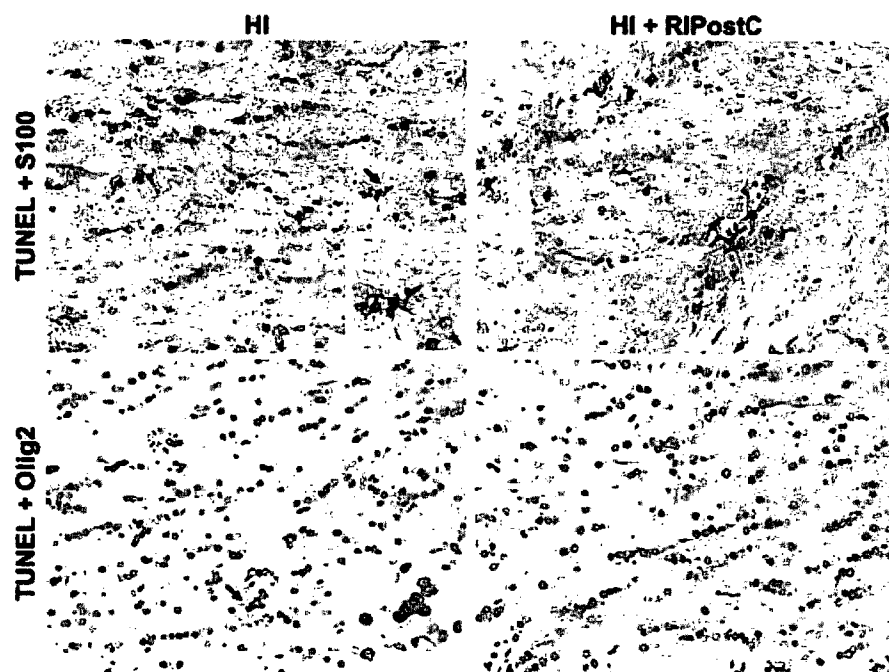


Figure 13

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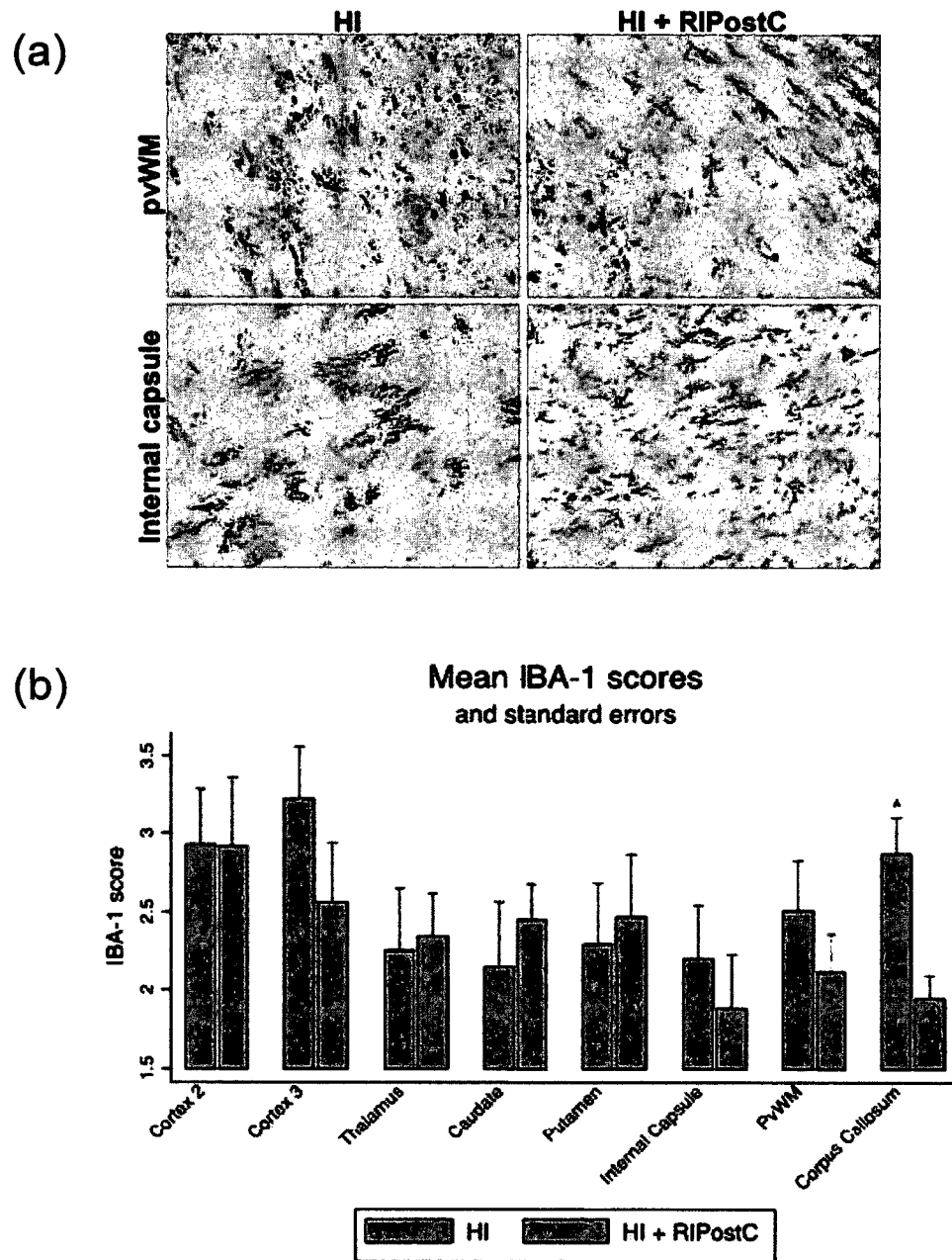
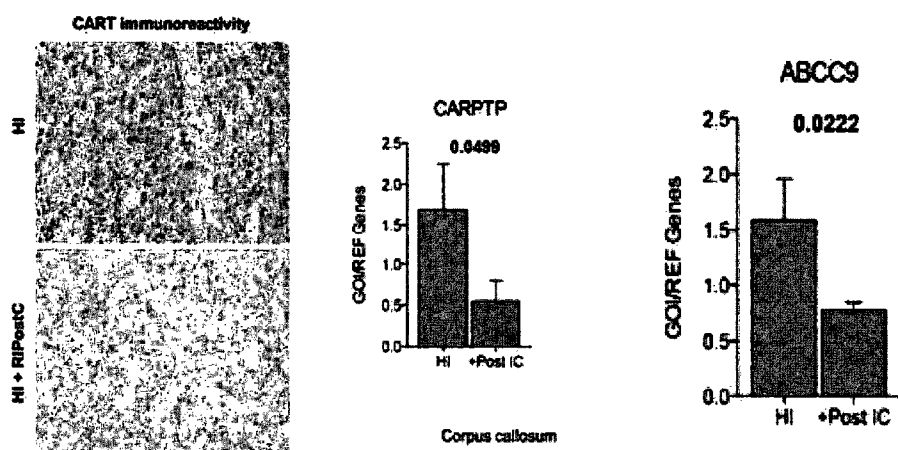


Figure 14

15/18



16/18

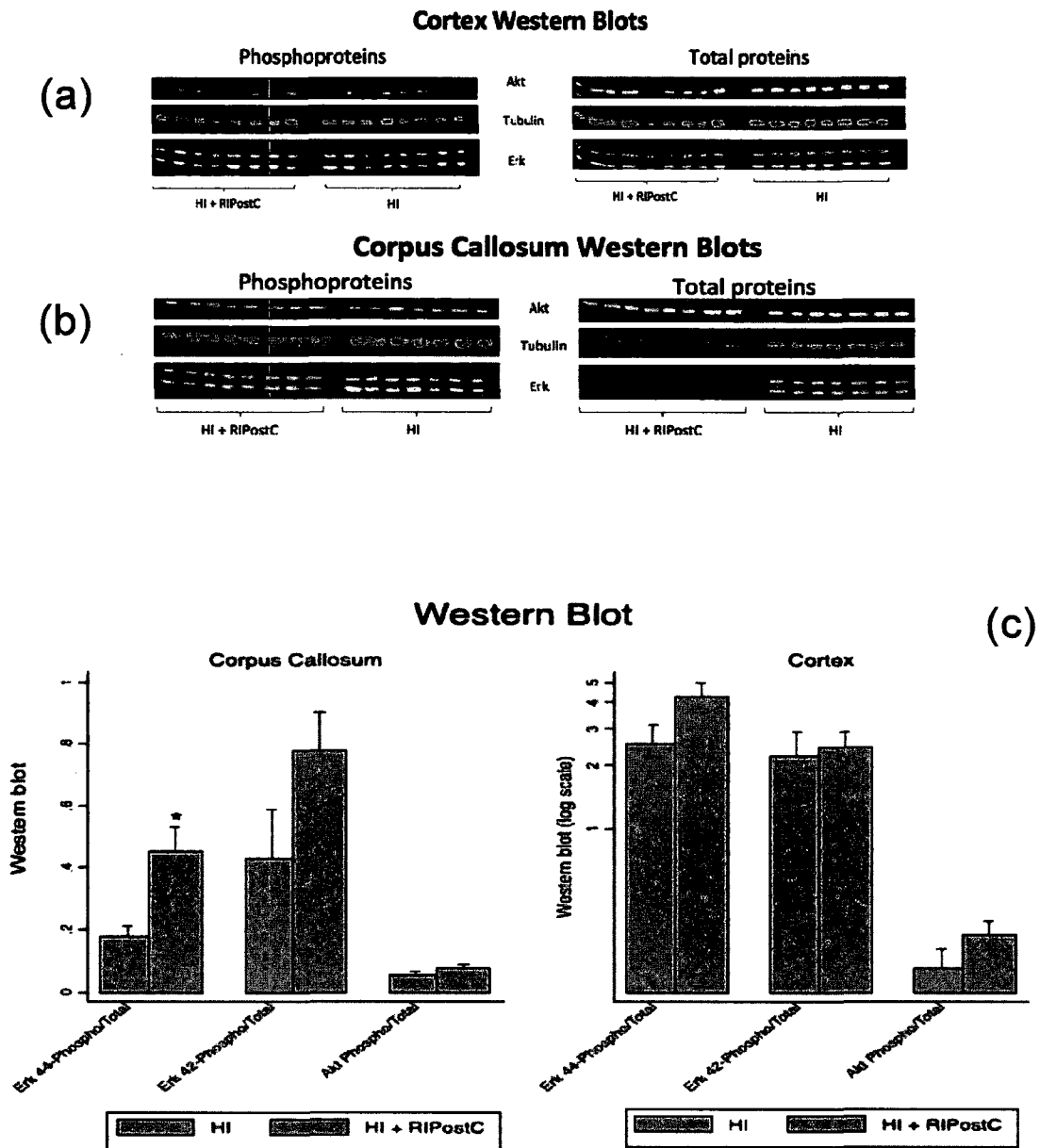


Figure 16

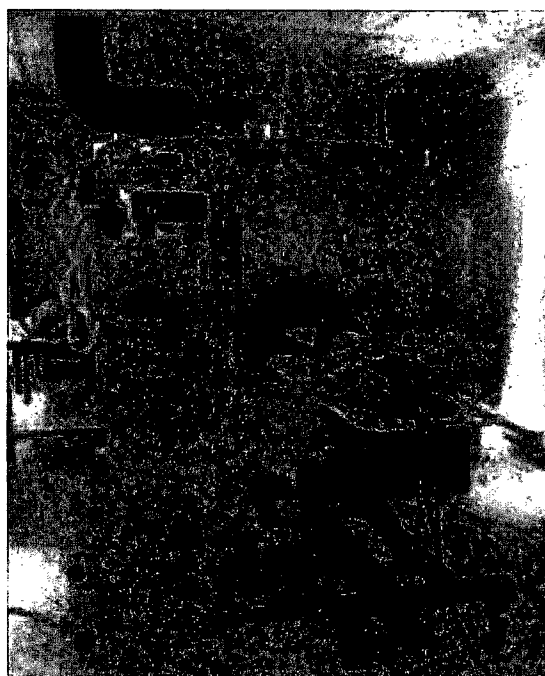
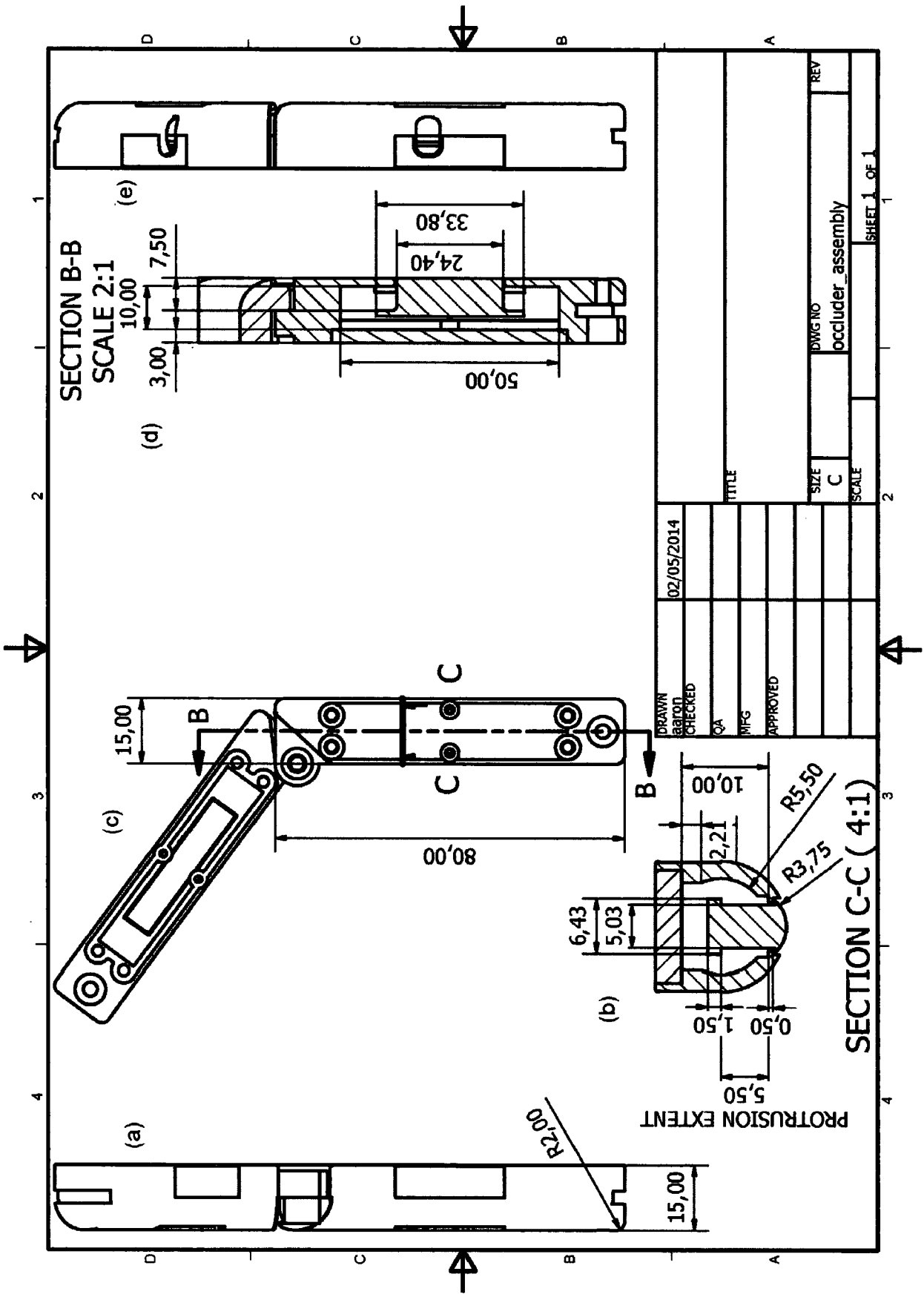


Figure 17

Figure 18



INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/051295

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61B17/132 A61B17/135 A61H9/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61B A61H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 201 426 747 Y (QI Y) 24 March 2010 (2010-03-24) the whole document -----	1-11, 13-16, 23,24
X	CN 201 551 362 U (YAFENG QI) 18 August 2010 (2010-08-18) the whole document -----	1-11, 13-16, 23,24
X	DE 40 12 974 A1 (DIMITROV PENTCHO [DE]) 31 October 1991 (1991-10-31) column 4, line 11 - column 7, line 9; figures ----- -/--	1-5, 9-11, 13-16, 23,24

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 June 2015

Date of mailing of the international search report

02/07/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Croatto, Loredana

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/051295

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/190026 A1 (SANDERS LAURIE [US]) 24 August 2006 (2006-08-24) paragraph [0027] - paragraph [0038]; figures -----	1-3, 9-11, 13-16, 23,24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2015/051295

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17-22, 26
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
2. ☒ Claims Nos.: 25
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Claims Nos.: 17-22, 26

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Continuation of Box II.2

Claims Nos.: 25

Claim 25 does not contain any technical features which can be searched. The scope of the claim is unclear (Article 6 PCT).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2015/051295

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
CN 201426747	Y	24-03-2010	CN 201426747 Y	24-03-2010
			WO 2010102426 A1	16-09-2010

CN 201551362	U	18-08-2010	NONE	

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