UNIVERSITÀ DEGLI STUDI DI TORINO

Doctoral School in Life and Health Sciences

PhD in Medical Pathophysiology

Cycle XXXIV

PhD THESIS

"Autologous Mitochondria Transplant as Therapeutic Model in the Treatment of Ischemia/Reperfusion Injury in Kidney Transplantation"

Scientific Supervisor

Professor Benedetta Bussolati

PhD Student

Dr. Andrea Rossi

Index

Abstract

Mitochondria are widely considered as the powerhouse of the cell, producing ATP and regulating the energy balancing. This very simple definition could shadow the importance of these organelles. Mitochondria, indeed, are fundamental for a plethora of functions, including apoptosis control and immune system regulation. The transfer of mitochondria among cells is a fascinating phenomenon triggered by stress signals, through which eukaryotic cells acquire new mitochondria from healthy donor cells, to ensure repair and survival after damage. During this process, the mitochondria's small size and capacity to change their shape and length, allow them to be transported from donor to recipient cells by transporting mechanisms like tunneling nanotubes and microvesicles. The organelles can be incorporated into the disrupted mitochondrial network of the host cells, restoring the energy supply, diminishing the reactive oxygen species produced, and eventually re-balancing the cell homeostasis. As mitochondria transfer has been found to play a critical role in healing several diseases among which brain and cardiac injury, respiratory and renal disorders, artificial mitochondria transfer (AMT) has recently emerged as a promising therapeutic approach for conditions characterized by mitochondrial damage, including ischemia-reperfusion injury (IRI). In the IRI syndrome, hypoxia triggers a cascade of events that will eventually hit the mitochondria and may lead to cellular necrosis if not adequately treated. IRI is particularly destructive in organ transplantation, which can ultimately lead to organ discard and delayed transplantation.

In the first part of the present thesis, we will expose the function and characteristics of mitochondria and the mechanisms of mitochondria-related toxicity in the pathological context. Subsequently, we will describe the mechanisms of mitochondria transfer among cells and the artificial mitochondria transfer, depicting the preclinical application of this treatment. Mitochondria transfer will be then inserted in the context of regenerative medicine, with our review: "extracellular vesicles, apoptotic bodies, and mitochondria: stem cell bioproducts for organ regeneration".

In the second part of the thesis, we will illustrate the mechanism of kidney preservation. In particular, we will focus on static cold storage, hypothermic machine perfusion, and normothermic machine perfusion. In the book chapter we wrote called “mitochondria transplantation in organ damage and repair” we described mitochondrial transfer in the context of organ repairment and tissue restoration. The chapter is part of the book titled: “Organ Repair and Regeneration: Preserving Organs in the Regenerative Medicine”.

Lastly, the third part of the thesis will be focused on my Ph.D. project. In this study, we investigated AMT as a possible therapeutic approach in the preservation and protection of transplanted organs, both *in vitro* and *ex vivo*. *In vitro*, we demonstrated that mitochondria transfer resulted in capable revert the deleterious effect of the ischemia reperfusion, whereas *ex vivo*, in a porcine model of kidney preservation, we were able to prove that AMT partially protects from ischemic damage.

1. FUNCTIONS AND CHARACTERISTIC OF MITOCHONDRIA

General Structure of Mitochondria

Mitochondria are usually defined as “membrane-bound organelle found in the cytoplasm of almost all eukaryotic cells, the primary function of which is to generate large quantities of energy in the form of ATP” [1]. Nevertheless, this definition is somehow limiting, shadowing the complexity of all the biological functions exerted by these organelles. The presence of their own genetic materials, the extensive interconnection with the other organelles, and the possibility to be shared and transferred between cells make mitochondria fundamental for a wide range of different roles. As matter of fact, mitochondrial disorders encompass almost all the fields of medicine, causing symptoms in any organ and presenting at any age [2].

Mitochondria are considered unique organelles also for the peculiar origin. According to the most accepted theory, the *endosymbiotic theory*, mitochondria originated from the endosymbiosis with a precursor procaryotic cell, similar to a modern bacteria, capable of oxidative phosphorylation [3]. This transition to permanent organelle determined an enormous number of evolutionary changes, from the origin of hundreds of new genes, to the integration of metabolism and reproduction [4]. However, other conserved organelle components, such as the peculiar DNA or various proteins similar in structure to the bacterial ones, not only

are not tolerated by the innate immune system, but are considered to be essential regulator of cell danger responses, acting at all steps of inflammatory responses [5].

Mitochondria are usually oval in shape and range in size from 0.5 to 10 μm. The numbers of mitochondria can vary enormously among the different cell types: from none in the erythrocytes, to hundreds of thousands in myocytes and hepatocytes. Due to the tendency of these organelles to fuse and dissociate, usually is difficult or even impossible to quantify a definite number. For this reason speaking about mitochondrial network, more than single mitochondria, results more appropriate [6]. Other than the production of energy, mitochondria are considered as the trigger of the so-called intrinsic apoptosis pathway, mainly through the release of cytochrome C. Other functions include the regulation of cellular proliferation and differentiation, storage of calcium ions, heme synthesis reactions, and immunological signalling. Furthermore, in recent years, the transfer of mitochondria between cells emerged as a new mechanism, mainly related to cell repairment, cell reprogramming, or immune interchange.

In the next part of this section, the structure of mitochondrion, peculiar and fundamental for most of the mentioned functions, will be briefly described.

Mitochondrial membranes

Mitochondria are formed by an outer and inner membrane with different properties, an intermembrane space, the cristae, that extend the surface of the inner membrane, and a matrix, which is fluid (fig. 1). The outer mitochondrial membrane enclosed the entire organelle and contain a massive amount of

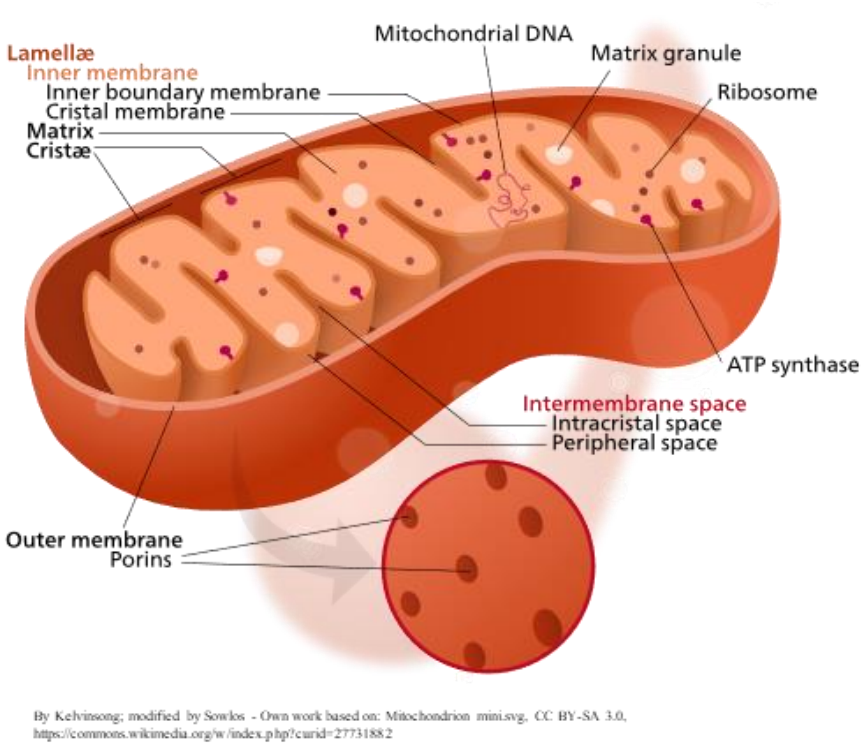
integral membrane proteins, including porins and voltage-dependent anion channels (VDAC). Interestingly, VDAC, which is the primary transporter of nucleotides, ions, and metabolites between cytosol and mitochondria, forms a complex similar to the one observed in gram-negative bacteria [7]. The disruption of the outer mitochondria barrier, with consequent exposure of the intermembrane space proteins has been associated with the trigger of programmed cell death [8]. The space between the outer and inner membrane, called perimitochondrial space, contains a series of proteins fundamental for the mitochondria functions, including cytochrome C [9]. The inner membrane, differently from the outer, is highly impermeable, and almost all molecules and ions need special transporters to pass through it. Another important characteristic is the compartmentalization in folds called cristae. Cristae highly expand the surface of the inner membrane, increasing the production of ATP. The most internal part of the mitochondria is the matrix, which contains most of the proteins of the mitochondrion, including the ATP synthase. Other important molecules included in the matrix are the mitochondrial ribosomes, different from the cytosolic ones, tRNA, and the mitochondrial DNA [10].

Mitochondrial DNA

Mitochondria are the only organelle to contain their own chromosome called mitochondrial DNA, or mtDNA. mtDNA is a small, double-stranded circular molecule containing the sequence of 37 genes with several unique features that differentiate it with nuclear DNA [11] (fig. 1). Firstly, only 13 of the ~ 1200 protein normally present in the mitochondrion are encoded from mtDNA. The

others, including the ones involved in the expression and maintenance of mtDNA, are nuclear gene products [12]. This characteristic is correlated to the separated evolutionary origin of mitochondria and eucaryotic cell. Most of the protein present in the mitochondria and not encoded by mtDNA are thought to have originally been of bacterial origin, having since been transferred to the eukaryotic nucleus during evolution. Moreover, the numbers of mtDNA copies present in each cell seems to be in excess of what is needed to sustain the normal functions of mitochondrion. This amount can be regulated through specific pathways controlled by cell-stress induction, which suggest an evolutionary pressure to maintaining high mtDNA copies, possibly related to mitochondrial signalling and/or immune functions [12], [13]. Another important characteristic is the peculiar methylation path of mtDNA, probably a remnant of the bacterial origin. Mutations in mtNDA occurs much more frequently than nuclear DNA. This can be due to close proximity of the genome to the ECT complex, and thus to a large amount of ROS, and by the absence of an efficient DNA repairment system [14]. Accumulation of mutations of mtDNA has been reported to be correlated with aging, but also with neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases [15], [16]. These features make the mtDNA as an endogenous trigger of both pro-inflammatory and type I interferon (IFN) responses, [17], [18] as firstly demonstrated by Collins et al [19]. The increase of mtDNA, internalized in extracellular vesicles (EVs), a heterogeneous group of cell-derived membranous structures, and released in the extracellular environment can act as not only as trigger of immune response, but also as endothelial cell activator [20], [21].

Another essential and very well know characteristic of mtDNA is that, in sexual reproduction, it is inherited exclusively from the mother, a mechanism called uniparental inheritance. The cause could be correlated with the simple dilution, as an egg contains on average 200,000 mtDNA molecules, whereas a healthy human sperm has been reported to contain on average 5 molecules; but also with other mechanism, that involved the ubiquitination and destruction of the spermal mtDNA [22], [23].



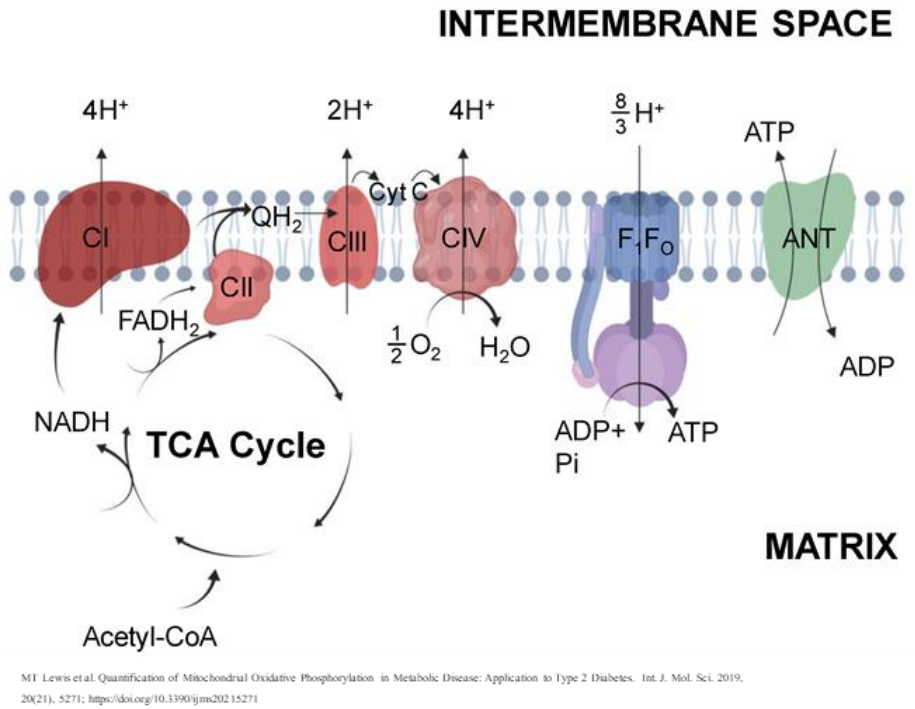
By Kelvinsong; modified by Sowlos - Own work based on: Mitochondrion mini.svg, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=27731882

Figure 1. The structure of mitochondrion comprehensive of outer membrane, inner membrane, matrix, and mitochondrial DNA.

Functions of mitochondria

As already mentioned, the principal and best-known function of mitochondria are to produce energy through the oxidative phosphorylation (OXPHOS), a complex series of biochemical reactions by which pyruvate, one of the final results of glycolysis, is oxidized to produce cofactors that drive the electron transport chain (ETC), generating ATP. Pyruvate is transported through the inner mitochondria membrane where can be oxidized and combined with coenzyme A, to form acetyl-CoA, or carboxylated to form oxaloacetate. These molecules are the starting point of the tricarboxylic acid cycle (TCA) (also called Krebs cycle) a series of chemical reactions able to release the stored energy of acetyl-CoA, producing energy, CO_2, and water (fig. 2). The Oxidation of acetyl-CoA is responsible for the production of nearly 2/3 of the total energy consumed by the body. In addition, the TCA cycle provides precursors of various amino acids, as well as NADH and $FADH_2$, used in several other pathways. The three molecules of NADH and the one $FADH_2$ produced are transferred to oxygen, an energy-rich molecule, increasing the concentration of protons in the intermembrane space. The potential energy of the proton gradient is exploited by the ATP synthase to generate ATP from ADP and inorganic phosphate (Pi) [24] (fig. 2).

Other from the ATP production, the gradient generated through OXPHOS is used in several other pathways, such as proteins import or trigger molecular changes in response to stress or mitochondria disfunction [25]. The ROS produced from ETC, instead, contributed to the control of cell proliferation, cell differentiation but also



MT Lewis et al. Quantification of Mitochondrial Oxidative Phosphorylation in Metabolic Disease: Application to Type 2 Diabetes. Int. J. Mol. Sci. 2019, 20(21), 5271; https://doi.org/10.3390/ijms202 15271

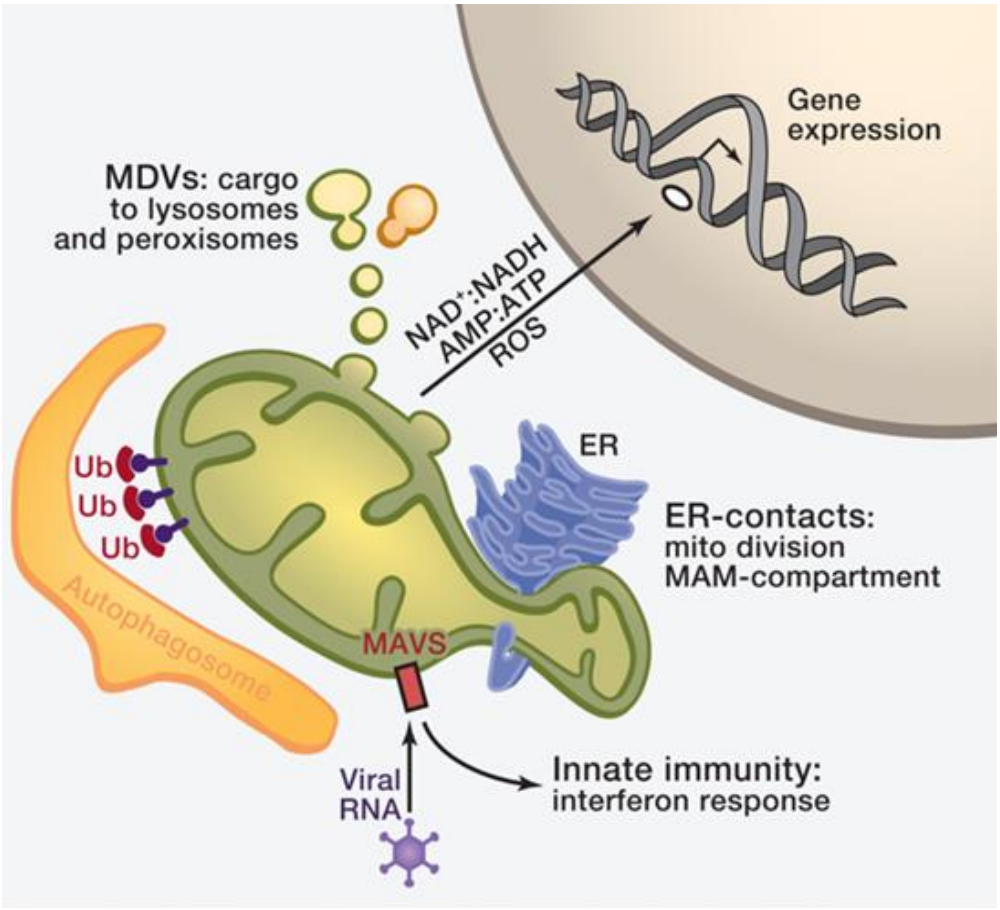
Figure 2. Schematization of the electron transport chain (ETC) in the mitochondrial intermembrane space. The NADH and FADH2 produced through TCA cycle are used to generate a H+ gradient in the intermembrane space. The ATP synthase use this gradient to convert ADP and Pi in ATP.

to adaptive stress signalling pathway, as hypoxia response [26]. In the neurons, the control of Ca^{2+} efflux is essential for modulating neurotransmitter release, neurogenesis, and neuronal plasticity. Moreover, the surplus of ATP is used to produce neurotransmitter as GABA and glutamate [27].

Mitochondria shape and dynamics are intimately correlated with their functions, in particular, as response to programmed cell death. The reorganization of mitochondrial cristae and crista junctions (tubules that connect the cristae to the

boundary) facilitate the release of proapoptotic molecules in the intermembrane space. The lateral reorganization of the outer membrane is used as signalling platform for pathways such as BCL-2 protein-dependent apoptosis, or innate immunity, though mechanism not fully understood [28], [29].

Mitochondria are strictly interconnected with other organelles, such as plasma membrane, peroxisomes, ER, autophagosome, and lysosomes (fig. 3). The interoganelles communication is ensured through direct membrane connection, and lysosomes, the so-called mitochondria-associated membranes (MAMs); or through intracellular EVs, extroflected directly from mitochondria. The treatment with antimycin A, a molecule able to block the complex III and is used to simulate ischemia damage, stimulate the EVs release from mitochondria directed to the liposome, suggesting that this mechanism could act as a quality control pathway for mitochondria homeostasis [30]. Mitochondrial disfunction, however, can influence not only the tissue, but also the organ and whole the organism. For instance, in mice models of mitochondria related disease as well as in human patients, OXPHOS deficient skeletal muscle secrete the fasting-related hormone FGF21. This protein can enter in the blood stream, inducing ketogenesis in the liver and mobilizes lipids from adipose tissue for oxidation [31], [32].



J. Nunnari et al. Mitochondria: In Sickness and in Health. Cell 148, March 16, 2012. DOI 10.1016/j.cell.2012.02.035

Figure 3. Interorganellar communication among autophagosome, ER and nucleus. Mitochondria-derived vesicles (MDV) are produced in response to organelle-to-organelle communication.

References

[1] K. Rogers, “mitochondrion | Definition, Function, Structure, & Facts | Britannica,” *Encyclopedia Britannica*, 2020. https://www.britannica.com/science/mitochondrion (accessed Sep. 09, 2021).

[2] E. Ylikallio and A. Suomalainen, “Mechanisms of mitochondrial diseases,” *Ann. Med.*, vol. 44, no. 1, pp. 41–59, 2012, doi: 10.3109/07853890.2011.598547.

[3] S. L, “On the origin of mitosing cells,” *J. Theor. Biol.*, vol. 14, no. 3, 1967, doi: 10.1016/0022-5193(67)90079-3.

[4] A. J. Roger, S. A. Muñoz-Gómez, and R. Kamikawa, “The Origin and Diversification of Mitochondria,” *Current Biology*, vol. 27, no. 21. Curr Biol, pp. R1177–R1192, Nov. 06, 2017, doi: 10.1016/j.cub.2017.09.015.

[5] A. Meyer *et al.*, “Mitochondria: An Organelle of Bacterial Origin Controlling Inflammation,” *Front. Immunol.*, vol. 0, no. APR, p. 536, Apr. 2018, doi: 10.3389/FIMMU.2018.00536.

[6] H. Hoitzing, I. G. Johnston, and N. S. Jones, “What is the function of mitochondrial networks? A theoretical assessment of hypotheses and proposal for future research,” *BioEssays*, vol. 37, no. 6, pp. 687–700, Jun. 2015, doi: 10.1002/bies.201400188.

[7] J. M. Herrmann and W. Neupert, “Protein transport into mitochondria,” *Current Opinion in Microbiology*, vol. 3, no. 2. pp. 210–214, 2000, doi: 10.1016/S1369-5274(00)00077-1.

[8] M. Červinka, “The role of mitochondria in apoptosis induced in vitro,” *Gen. Physiol. Biophys.*, vol. 18, no. SPEC. ISS., pp. 33–40, 1999, doi: 10.1146/annurev-genet-102108-134850.The.

[9] V. G. A. Arciuch, M. E. Elguero, J. J. Poderoso, and M. C. Carreras, “Mitochondrial regulation of cell cycle and proliferation,” *Antioxidants and Redox Signaling*, vol. 16, no. 10. pp. 1150–1180, May 15, 2012, doi: 10.1089/ars.2011.4085.

[10] T. G. Freya and C. A. Mannellab, “The internal structure of mitochondria,” *Trends in Biochemical Sciences*, vol. 25, no. 7. Trends Biochem Sci, pp. 319–324, Jul. 01, 2000, doi: 10.1016/S0968-0004(00)01609-1.

[11] S. GS and C. DA, “Mitochondrial DNA maintenance in vertebrates,” *Annu. Rev. Biochem.*, vol. 66, pp. 409–436, 1997, doi: 10.1146/ANNUREV.BIOCHEM.66.1.409.

[12] N. D. Bonawitz, D. A. Clayton, and G. S. Shadel, “Initiation and Beyond: Multiple Functions of the Human Mitochondrial Transcription Machinery,” *Mol. Cell*, vol. 24, no. 6, pp. 813–825, Dec. 2006, doi: 10.1016/J.MOLCEL.2006.11.024.

[13] A. P. West and G. S. Shadel, “Mitochondrial DNA in innate immune responses and inflammatory pathology,” *Nat. Rev. Immunol.*, vol. 17, no. 6, pp. 363–375, 2017, doi: 10.1038/nri.2017.21.

[14] L. A. Zinovkina, “Mechanisms of Mitochondrial DNA Repair in Mammals,” *Biochem.*, vol. 83, no. 3, pp. 233–249, 2018, doi: 10.1134/S0006297918030045.

[15] L. MT *et al.*, “Somatic mitochondrial DNA mutations in early Parkinson and incidental Lewy body disease,” *Ann. Neurol.*, vol. 71, no. 6, pp. 850–854, Jun. 2012, doi: 10.1002/ANA.23568.

[16] K. Y, K. E, M. AC, G. C, K. NW, and K. K, “Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons,” *Nat. Genet.*, vol. 38, no. 5, pp. 518–520, May 2006, doi: 10.1038/NG1778.

[17] W. AP, S. GS, and G. S, “Mitochondria in innate immune responses,” *Nat. Rev. Immunol.*, vol. 11, no. 6, pp. 389–402, Jun. 2011, doi: 10.1038/NRI2975.

[18] S. E. Weinberg, L. A. Sena, and N. S. Chandel, “Mitochondria in the regulation of innate and adaptive immunity,” *Immunity*, vol. 42, no. 3. Immunity, pp. 406–417, Mar. 17, 2015, doi: 10.1016/j.immuni.2015.02.002.

[19] C. LV, H. S, H. E, J. IM, and T. A, “Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses,” *J. Leukoc. Biol.*, vol. 75, no. 6, pp. 995–1000, Jun. 2004, doi: 10.1189/JLB.0703328.

[20] K. P. Hough *et al.*, “Exosomal transfer of mitochondria from airway myeloid-derived regulatory cells to T cells,” *Redox Biol.*, vol. 18, no. June, pp. 54–64, 2018, doi: 10.1016/j.redox.2018.06.009.

[21] M. Tong *et al.*, “Antiphospholipid antibodies increase the levels of mitochondrial DNA in placental extracellular vesicles: Alarmin-g for preeclampsia,” *Sci. Rep.*, vol. 7, no. 1, pp. 1–12, 2017, doi: 10.1038/s41598-017-16448-5.

[22] P. Sutovsky, R. D. Moreno, J. Ramalho-Santos, T. Dominko, C. Simerly, and G. Schatten, “Ubiquitin tag for sperm mitochondria,” *Nat. 1999 4026760*, vol. 402, no. 6760, pp. 371–372, Nov. 1999, doi: 10.1038/46466.

[23] H. B. H. C. D. W. RE Giles, “Maternal inheritance of human mitochondrial DNA,” *Proc. Natl Acad. Sci. USA*, vol. 77, no. 11 I, pp. 6715–6719, 1980, doi: 10.1073/pnas.77.11.6715.

[24] M. Akram, “Citric Acid Cycle and Role of its Intermediates in Metabolism,” *Cell Biochemistry and Biophysics*, vol. 68, no. 3. Cell Biochem Biophys, pp. 475–478, 2014, doi: 10.1007/s12013-013-9750-1.

[25] P. Bhargava and R. G. Schnellmann, “Mitochondrial energetics in the kidney,” *Nature Reviews Nephrology*, vol. 13, no. 10. Nature Publishing Group, pp. 629–646, Oct. 01, 2017, doi: 10.1038/nrneph.2017.107.

[26] H. RB and C. NS, “Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes,” *Trends Biochem. Sci.*, vol. 35, no. 9, pp. 505–513, Sep. 2010, doi: 10.1016/J.TIBS.2010.04.002.

[27] N. R. Sibson, A. Dhankhar, G. F. Mason, D. L. Rothman, K. L. Behar, and R. G. Shulman, “Stoichiometric coupling of brain glucose metabolism and glutamatergic neuronal activity,” *Proc. Natl. Acad. Sci.*, vol. 95, no. 1, pp. 316–321, Jan. 1998, doi: 10.1073/PNAS.95.1.316.

[28] C. DH, N. T, and L. SA, “Mitochondrial dynamics in cell death and neurodegeneration,” *Cell. Mol. Life Sci.*, vol. 67, no. 20, pp. 3435–3447, Oct. 2010, doi: 10.1007/S00018-010-0435-2.

[29] J. E. Chipuk, T. Moldoveanu, F. Llambi, M. J. Parsons, and D. R. Green, “The BCL-2 Family Reunion,” *Mol. Cell*, vol. 37, no. 3, p. 299, Feb. 2010, doi: 10.1016/J.MOLCEL.2010.01.025.

[30] N. M *et al.*, “Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers,” *Curr. Biol.*, vol. 18, no. 2, pp. 102–108, Jan. 2008, doi: 10.1016/J.CUB.2007.12.038.

[31] M. K. Badman, A. Koester, J. S. Flier, A. Kharitonenkov, and E. Maratos-Flier, “Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis,” *Endocrinology*, vol. 150, no. 11, pp. 4931–4940, Nov. 2009, doi: 10.1210/en.2009-0532.

[32] R. H. Baloh, R. E. Schmidt, A. Pestronk, and J. Milbrandt, “Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations,” *J. Neurosci.*, vol. 27, no. 2, pp. 422–430, Jan. 2007, doi: 10.1523/JNEUROSCI.4798-06.2007.

2. TRANSFER OF MITOCHONDRIA AMONG CELLS

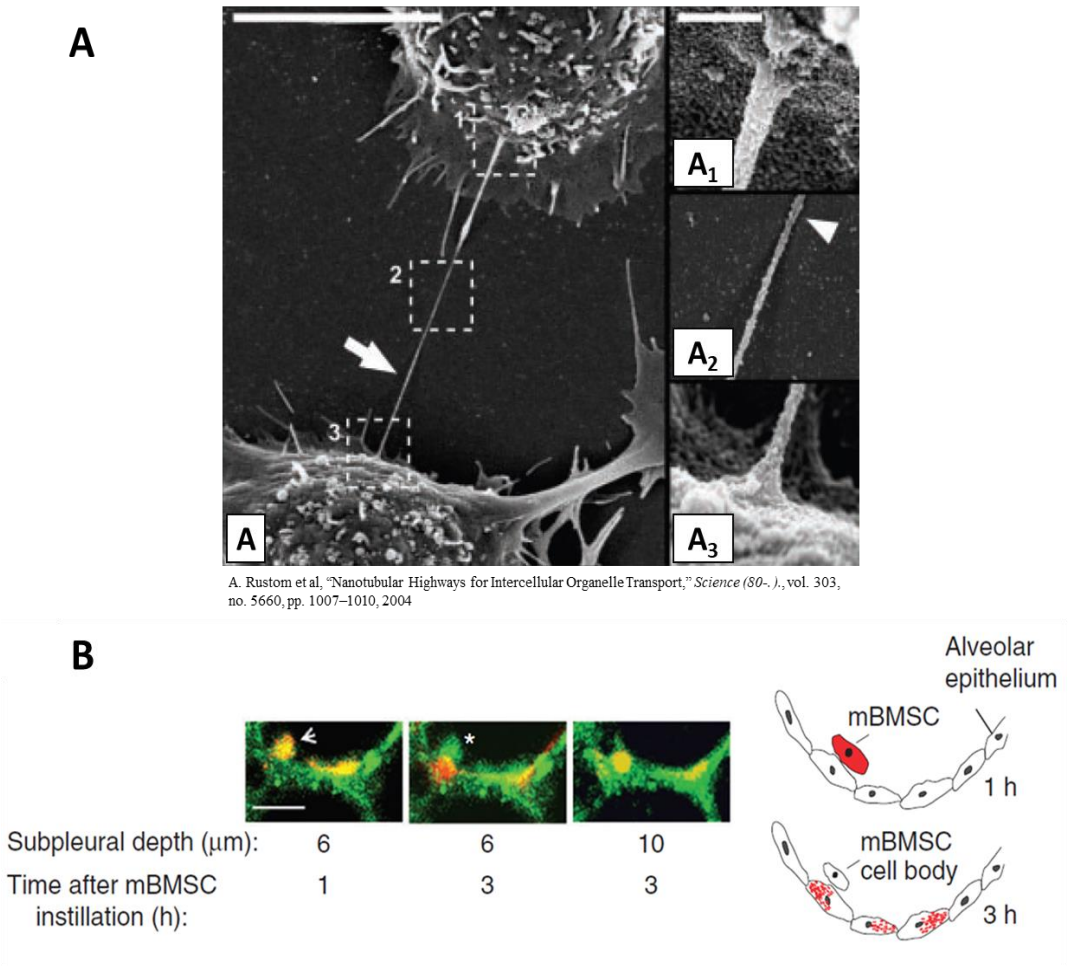
Intracellular Mitochondrial Transfer

The previous chapter highlighted the pivotal importance of mitochondria for the correct function of the cell, and how mitochondrial dysfunction can lead to cell death, activate the immune system response and, eventually, induce the clinical progression of a pre-existent pathology. As a matter of fact, mitochondria do not possess an efficient repair system and even small alterations in the mitochondrial network can influence the cell fate. In order to restore the mitochondrial homeostasis, or balance the intracellular energetic supply, mitochondria can be transferred from one part of the cell to another. This intracellular transfer of mitochondria has been mostly observed in very polarized cells, such as neurons having long axons. Mitochondria can be relocated from the cell body to the periphery, in the region of synaptic contacts, as response to high energy expense [1], [2]. Another case of intracellular mitochondria transfer has been studied in cells where the area adjacent to the blood flow, and thus better provided with nutrients and oxygen, is far away from the part with higher energy demand. The mitochondria inside these cells can be translocated, through a system of microtubules, acting as mobile power stations [3]. Rather than the balancing of energy supply, the transfer of mitochondria inside the cell can be used to redirect impaired mitochondria to autophagosomes. Disorders in the intracellular mitochondria transfer have been associated with neurological diseases, such as Alzheimer's disease [4].

Physiological Mitochondria Transfer

In recent years, the studies on intracellular mitochondria transfer were complemented with a new mechanism of intercellular mitochondria repairment, where the mitochondria are delivered from one cell to another; a mechanism called *physiological mitochondrial transfer* (PMT). Most of the studies have linked the PMT with the repair of the damaged host cell by healthy cells, such as mesenchymal stem cells (MSCs).

The first pieces of evidence of a transfer of mitochondria between cells emerged in 2004 with the work of Rustom et al., where the transfer occurred through the establishment of long protrusion of cytoplasm called tunneling nanotubes (TNTs) [5] (fig. 4, A). In 2005, Koyanagi's lab. demonstrated that mitochondria are transported by TNTs from human endothelial progenitor cells to neonatal undifferentiated cardiomyocytes. According to their piece of evidence, this process is important for cardiomyocytes differentiation [6]. One year later, Spees et al. demonstrated, for the first time, the rescue of mitochondria-impaired cells through the transfer of mitochondria or mtDNA. In this work, cells were cultured and treated with ethidium bromide, in order to damage mtDNA, impairing aerobic respiration and growth. The coculture with human MSCs (hMSCs) derived from bone marrow or skin fibroblast was able to restore aerobic respiration. Interestingly, after coculture, the experiments indicated the presence of functional and active mitochondria [7].



A. Rustom et al, "Nanotubular Highways for Intercellular Organelle Transport," *Science (80-.).*, vol. 303, no. 5660, pp. 1007–1010, 2004

D. G. Phinney *et al.*, "Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs," *Nat. Commun.*, vol. 6, pp. 1–15, 2015.

Figure 4. A. Architecture of TNT between two cultured cells by SEM. For boxed areas, higher magnification images are shown. **B.** Left, mBMSC (arrow) transfering red-labelled mitochondria on the adjacent alveolar epithelium (green). Right, the illustration depicts the imaging data on the left at two time points. Mitochondria are shown in red, and the mBMSC cell body is marked by an asterisk.

In 2012, the work of Islam et al. shed new light on our comprehension of PMT. They observed that, in a mouse model of LPS-induced acute lung injury, the administration of human bone marrow–derived MSCs (BN-MSCs) reverted leucocytosis and protein leak, in concomitance of a marked increase in the ATP production. This rescue was associated with the release of mitochondria containing EVs that the lung epithelia engulfed (fig. 4, B). The presence of exogenous mitochondria was demonstrated optically, and through the detection of human mtDNA in the lung epithelial cells. Interestingly, the beneficial effect was abrogated by BN-MSCs with dysfunctional mitochondria [8]. In a similar manner, Cho et al. demonstrated that MSCs actively transferred healthy mitochondria by TNTs to mitochondria-depleted cells. To prove this, MSCs were treated with rhodamine 6G in order to impair mitochondrial function but not mtDNA. Treated MSCs failed to rescue the aerobic respiration of mitochondria free cells [9]. EVs transfer of mitochondria has been associated, also, with the maintenance of cell homeostasis and cell-to-cell communication. Phinney et al. linked mitophagy, a particular form of autophagy that involves mitochondria, with the transfer of mitochondria to macrophages. The internalization of mitochondria enriched EVs produced by MSCs increased the energy production of macrophages. At the same time, they observed that the exchange of microRNA between macrophages and MSCs downregulated TLR expression and inflammatory signaling, suggesting the presence of tolerance systems used by host cells to allow the absorption and entrance of external mitochondria [10].

The nervous system is particularly susceptible to mitochondria disruption, and for this reason PMT has been extensively studied in neurons and astrocytes. Different studies reported that PMT allows cells to get rid of unfunctional mitochondria, replacing them with functional ones. This mechanism not only protects neuronal cell from damage but also allow the recycle these organelles in other cells, a process called transcellular degradation or transmitophagy. In transmitophagy, neurons tend to outward cellular structures rich in mitochondria; these structures are captured by astrocytes and the mitochondria recycled. The mechanisms behind transmitophagy are unclear, but some evidence suggests that damaged axons could stimulate the process. According to the work of Davis et al, the transport of mitochondria back in the axonal soma could be energetically disadvantageous, thus mitochondria are simply transferred to near astrocytes [11].

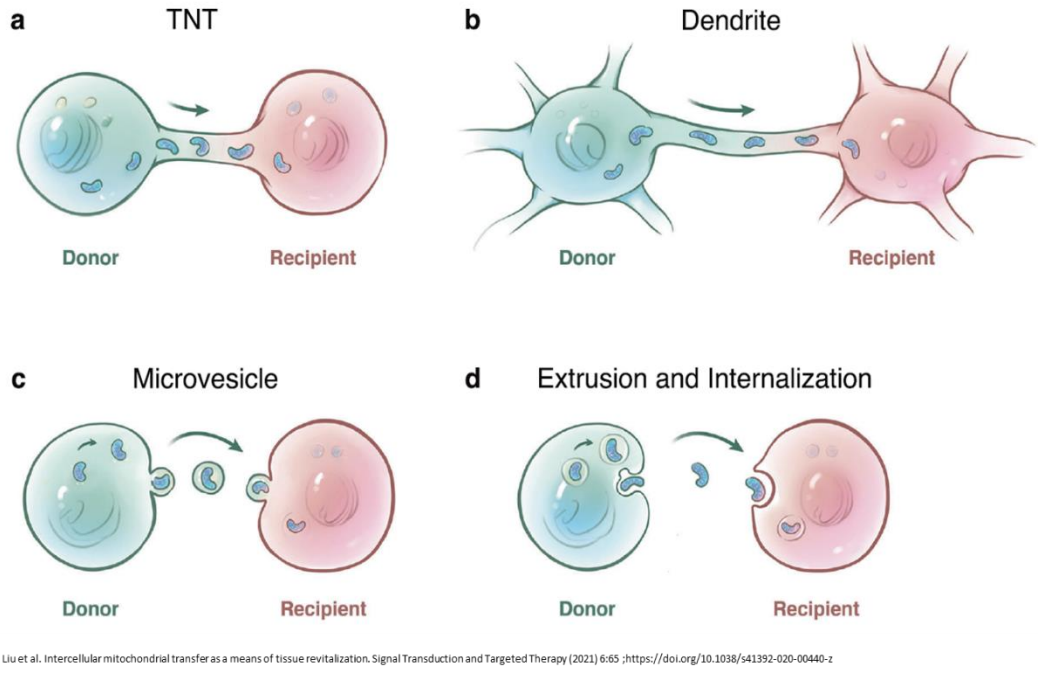
Other than TNTs and EVs, mitochondria can be transferred through extrusion in the extracellular microenvironment without any carrier, to be internalized by host cells. Mostly, this extrusion of mitochondria occurs as a quality control mechanism or as a danger signal when the cell is under stress. Nakajima et al. described that damaged mitochondria can be extruded from TNFα-induced dying cells by fusing with the plasma membrane in a caspase-dependent manner [12]. In some cases, free mitochondria extruded from stressed cells were also indicated as a special danger signal to provoke inflammatory responses.

In conclusion, the principal routes of PMT are:

- TNT, a membranous tubular protrusion that extends from the plasma membrane, with a variety of diameters between 50 and 1500 nm and lengths

from several tens to hundreds of microns. TNT is the most popular route of mitochondrial transfer between the connected cells (fig. 5 A).

- Dendrite is another form of the membranous protrusion. Some cells with dendrites (e.g., osteocytes) are connected to each other via intrinsic



D. Liu et al. Intercellular mitochondrial transfer as a means of tissue revitalization. Signal Transduction and Targeted Therapy (2021) 6:65 ;https://doi.org/10.1038/s41392-020-00440-z

Figure 5. Routes of physiological mitochondrial transfer. **A.** Tunnelling nanotubes (TNTs), long protrusion between a cell and another. **B.** Dendrites, cellular extrusions similar to TNTs. **C.** Microvesicles, formed by blebbing of the cellular plasma membrane. **D.** extrusion and internalization of mitochondria, without any carrier.

dendrites to form an intercellular network, which provides a highway for mitochondrial transfer (fig. 5 B).

- Microvesicles formed by blebbing of the cellular plasma membrane were reported as another route for mitochondrial transfer (fig. 5 C).
- Free mitochondria alone can be extruded or internalized without carriers, which provides a possible route for intercellular mitochondrial transfer (fig. 5 D).

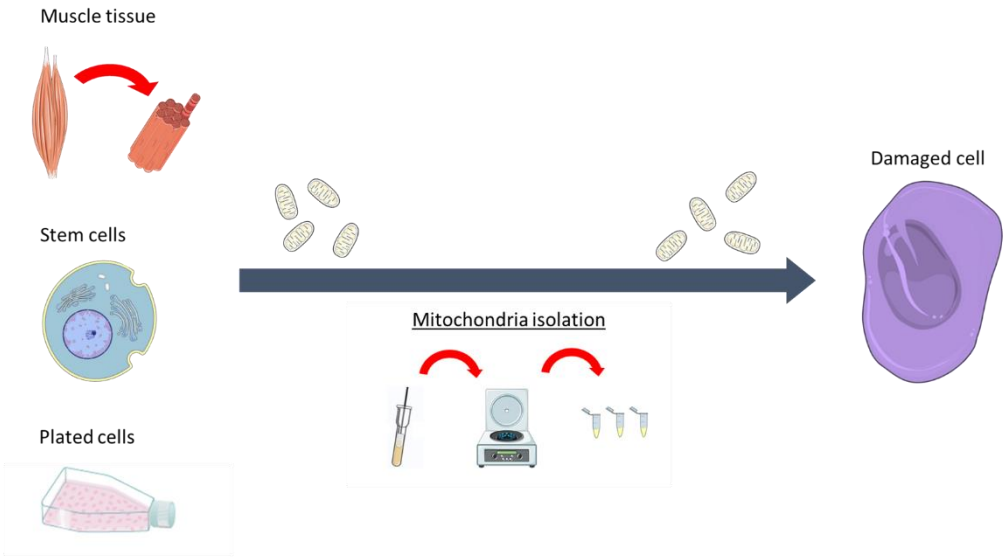


Figure 6. Graphical visualization of artificial mitochondrial transfer (AMT). Respiration-competent mitochondria can be isolated from different sources, as skeletal muscle tissue, stem cell, or from cultured cells. After a rapid isolation process, mitochondria are co-incubated with damaged cells in order to restore these cells or protect them from damage.

Artificial Mitochondrial Transfer

The artificial mitochondrial transfer (AMT), also named mitochondria transplant or mitochondrial transformation, is the use of mitochondria isolated from healthy cells as a possible mechanism of cellular repair or protection (fig. 6).

The very first transfer of mitochondria from one cell to another was conducted by Clark and Shay in 1982. They used the antibiotics chloramphenicol (CAP) and efrapeptin (EF), which inhibit the mitochondria's protein synthesis and ATPase function in order to kill sensitive fibroblasts. They, then, selected the fibroblast resistant to CAP and EF. The transfer of isolated mitochondria from CAP and EF-resistant fibroblasts through co-incubation increased the survival of the recipient cells, which were sensitive to these antibiotics. Mitochondria transferred from sensitive cells failed to confer resistance to recipient cells, excluding the possibility that higher mitochondria concentration could decrease CAF and EF damage. Interestingly, the failure of AMT with other cell types suggests that this process is not equally efficient among different cell types and that some cells may be more receptive than others [13]. In 1988, King and Attardi developed the first AMT technique using invasive instruments; they injected exogenous mitochondria isolated from CAP resistant cells into sensitive human cells. This study demonstrated that the injection of just one mitochondrion could very quickly repopulate a cell depleted of its endogenous mitochondria [14]. One of the first profs of the possible therapeutic potential of AMT came from the work of Pinkert et al.; they used microinjection to transfer mitochondria isolated from the livers of *Mus spretus* to fertilized oocytes taken from *Mus musculus*. After 4.5 days in

culture, Pinkert et al. detected xenogeneic mitochondria DNA sequences in the recipient cells, thereby demonstrating that xenogeneic mitochondria from closely related species are able to survive in recipient oocytes for at least a limited time [15]. In 2007, Yoon et al. demonstrated that mitochondria from different origins can fuse together, supporting the effectiveness of AMT. Firstly, they fused the cells with polyethylene glycol (PEG). Then, they labeled human and mice mitochondria differently (mtGFP and mtDsRed, resp.) and observed a mix of the two types of mitochondria 45 min after adding the PGE and fusion of the mitochondria of all hybrids at 4 h [16].

In order to improve the efficiency of AMT, various groups have tried to use chemical compounds and/or physical methods. In 2013, Margus et al. used Pep-1, a cell-penetrating peptide to foster mitochondria internalization. The mix of Pep-1 and isolated mitochondria increased the efficiency and the amount of mitochondria transferred [17]. Another method to increase AMT efficiency is the so-called mitoception created by Caicedo's group. This technique adds to AMT two other steps: centrifugation and a thermic shock. Mitoception allowed the constant and reproducible increase of mitochondrial uptake by the recipient cells proportionally to the material added. However, they observed that treated cells cannot constantly incorporate mitochondria without harming their functional properties, highlighting a possible functional threshold of the internalized mitochondria [18].

A series of evidence of the therapeutic potential of AMT *in vivo* came principally from the works of McCully's team. The rationale behind their works is that the replacement of affected mitochondria with healthy ones would significantly

improve recovery from ischemic injury. In 2009, McCully et al. proved for the first time that mitochondria can be used to repair damaged tissues in a rabbit model of IRI. They induced ischemia in the heart of rabbits by occluding the left coronary artery using the Langendorff perfusion allowing to test the contractile strength and heart rate. Then, they injected either a vehicle, vehicle with mitochondria or mitochondria alone which had been thawed after an overnight period at −20°C in the presence of the vehicle. These were injected directly into the ischemic zone just before reperfusion. Interestingly, they observed that the infarcted area was reduced, and the functional recovery increased after injecting mitochondria combined with the vehicle. This was not observed in the tissue only injected with mitochondria isolates, meaning that mitochondria must be active in order to serve therapeutic functions [19]. Furthermore, they reported the first

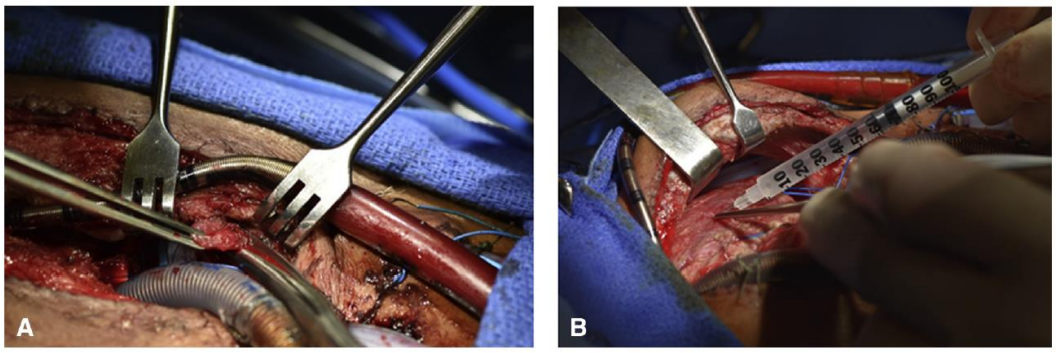


Figure 7. In the work of of Emani et al. a novel strategy to repair and replenish damaged mitochondria, termed mitochondrial autotransplantation, has been developed in which healthy autologous mitochondria harvested from nonischemic skeletal muscle are transplanted into injured myocardium. **A.** Biopsy of nonischemic skeletal muscle. **B.** Injection of autologous mitochondria into the myocardium with an insulin syringe.

clinical application of mitochondrial autotransplantation for myocardial recovery in pediatric patients who were supported by extracorporeal membrane oxygenation (ECMO) due to ischemia reperfusion injury. Although some issues concerning the dose and route of mitochondrial transplantation still need to be optimized, their primary results were encouraging, as four of the five patients showed improvement in their ventricular function and were successfully separated from ECMO support [20] (fig. 7).

The efficacy of AMT in the treatment of ischemia was further demonstrated by Masuzawa et al. They isolated mitochondria from the pectoral muscle of the same rabbit used for the ischemic shock (autologous transfer). After a follow-up of 28 days, infarct marker levels decreased and the generation of precursor metabolites for energy and cellular respiration increased. Interestingly, they also found that mitochondria were internalized by the cardiomyocytes 2 hours after transplantation, with cardioprotective effects after 28 days [21].

The mechanisms of internalization of isolated mitochondria during AMT have been extensively discussed, but, to date, they remain unclear. Kitani et al. and Kesner et al. proposed macropinocytosis as the basis for mitochondrial internalization [22], [23]. Macropinocytosis is a particular pathway of endocytosis characterised by the nonspecific internalisation of large amounts of extracellular fluid, solutes, and membranes in large endocytic vesicles. There are multiple pathways of macropinocytosis utilised by different cell types, and some of these pathways are

triggered by different stimuli. Notably, not all the cell types are able to do macropinocytosis, and that could partially explain why the AMT can vary among different cell types or tissues. McCully's team, however, observed that the transfer of mitochondria and their in vivo internalization was mediated by actin-dependent endocytosis and not by macropinocytosis. The use of different inhibitors to stop endocytosis and TNTs greatly decreased the uptakes process [24]. Further assays need to be developed *in vivo* to fully understand the process of internalization related to AMT, possible heterogeneity across different tissues, and the effects of the transfer of mitochondria to harmed tissue.

Methodology of Mitochondria Isolation and Characterization

A critical point of AMT is mitochondria isolation. Mitochondria, in order to be absorbed efficiently, must be viable and respiration competent. In fact, during and after isolation, these organelles are particularly susceptible to several physical and chemical factors. Freezing and thaw cycle tend to disrupt the outer membrane, and thus the mitochondria should be used "fresh" for each experiment. The isolation must be performed in a specific medium; conventionally, in a sucrose/mannitol-enriched isosmotic medium with a physiological pH. The temperature of extraction is a fundamental parameter. The isolation and storage at 4° C greatly improve the efficiency and the quality of the mitochondrial isolation. Another important parameter to consider is the quantity of mitochondria used for ATM. There is great heterogeneity in the literature regarding the quantity of mitochondria, signifying the importance to consider the influence cell-source could have on the number of

mitochondria used per host cell. For instance, McCully used $9.7 \times 10^6 \pm 1.5 \times 10^6$/ml of mitochondria isolated from healthy hearts in injections of 0.1 ml, eight times into the affected zone of the ischemic hearts [20]. Instead in their study, I-Rue Lai et al. used a concentration of $7.7 \times 106 \pm 1.5 \times 10^6$/ml of mitochondria isolated from healthy livers in one injection of 0.1 ml into the subcapsular region of the spleen poles [25]. Although each concentration yielded therapeutic benefits, neither was established as the optimal injection concentration.

As mentioned before, there are different techniques and methodologies to extract and characterize isolated mitochondria. In the next part of the chapter, the isolation protocols and the characterization methodologies will be briefly analysed.

Mitochondria Isolation Protocols

To date, there are several different protocols or commercial kits that can be used to isolate mitochondria for AMT. Interestingly, there are few differences between *in vitro* and *in vivo* isolation protocols and are mostly related to tissue homogenization and the preliminary isolation of cells. As mentioned, in order to develop an efficient protocol for mitochondria isolation suitable for AMT, a balance between the grade of purity and the timing of isolation must be assessed. In any case, low temperature is mandatory, and mitochondria must be transferred as soon as possible.

The great part of the available protocols begins with the scraping of the cells or the homogenization of the tissue. This phase must be a good compromise between homogenization of the cells and the preservation of mitochondrial

integrity. After this passage, a first centrifugation at low speed (usually 600-800g) is performed, in order to seed and thus eliminate all debris, membranes, and nuclei. Then, the supernatant is collected and centrifuged again at faster speed (7000 to 9000g). This centrifugation allows to pellet only mitochondria and similar size debris, eliminating all the other possible sources of contamination, including exosomes and microvesicles. The obtained pellet is resuspended in precooled *respiration buffer*, formed by various carbohydrates (usually glutamate, sucrose, or glucose) dissolved in water at physiologic pH. This buffer allows mitochondria to work in an extracellular environment and preserve their functional characteristic.

Isolated Mitochondria Characterization

The functional characterization of isolated mitochondria is fundamental for a valuable transfer of mitochondria. Notably, the characterization must be performed immediately after isolation, to protect their integrity; in fact, the -20/-80 °C storage is able to disrupt the external mitochondrial membrane.

One of the easiest ways to assess mitochondria viability is to marker them with Mitotracker. a fluorescent dye absorbed only by mitochondria with a differential in membrane potential. This potential allows mitochondria to work correctly and thus only viable mitochondria are able to retain the dye. However, mitotracker staining alone is not sufficient to determine the functional viability, because some dye could enter also in damaged mitochondria, with very low differential potential among the membranes.

For this reason, other analyses, as the ATP production evaluation, are required.

Other useful markers of isolated mitochondria viability are molecules produced, or consumed, by active mitochondria. The analysis of O_2 consumption is commonly used for this purpose, as well as the evaluation of malate or succinate, molecules produced during the TCA cycle.

Another technique to evaluate isolated mitochondria can be the morphological evaluation through transmission electron microscopy. To be identified as morphologically intact, mitochondria must result rounded, and the internal cristae must be well visible.

References

[1] D. B. Zorov *et al.*, "The mitochondrion as Janus Bifrons," *Biochem.*, vol. 72, no. 10, pp. 1115–1126, Oct. 2007, doi: 10.1134/S0006297907100094.

[2] J. Schonbach, C. Schonbach, and M. Cuénod, "Rapid phase of axoplasmic flow and synaptic proteins: An electron microscopical autoradiographic study," *J. Comp. Neurol.*, vol. 141, no. 4, pp. 485–497, 1971, doi: 10.1002/CNE.901410406.

[3] A. AA, B. LE, C. YS, S. VP, and Z. DB, "Coupling membranes as energy-transmitting cables. I. Filamentous mitochondria in fibroblasts and mitochondrial clusters in cardiomyocytes," *J. Cell Biol.*, vol. 107, no. 2, pp. 481–495, 1988, doi: 10.1083/JCB.107.2.481.

[4] B. SJ, "Mitochondrial alterations in Alzheimer's disease," *J. Alzheimers. Dis.*, vol. 9, no. 2, pp. 119–126, 2006, doi: 10.3233/JAD-2006-9204.

[5] A. Rustom, R. Saffrich, I. Markovic, P. Walther, and H. H. Gerdes, "Nanotubular Highways for Intercellular Organelle Transport," *Science (80-.).*, vol. 303, no. 5660, pp. 1007–1010, 2004, doi: 10.1126/science.1093133.

[6] M. Koyanagi, R. P. Brandes, J. Haendeler, A. M. Zeiher, and S. Dimmeler, "Cell-to-Cell Connection of Endothelial Progenitor Cells With Cardiac Myocytes by Nanotubes," *Circ. Res.*, vol. 96, no. 10, pp. 1039–1041, May 2005, doi: 10.1161/01.RES.0000168650.23479.0C.

[7] J. L. Spees, S. D. Olson, M. J. Whitney, and D. J. Prockop, "Mitochondrial transfer between cells can rescue aerobic respiration," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 5, pp. 1283–1288, 2006, doi: 10.1073/pnas.0510511103.

[8] M. N. Islam *et al.*, "Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury," *Nat. Med.*, vol. 18, no. 5, pp. 759–765, 2012, doi: 10.1038/nm.2736.

[9] Y. M. Cho *et al.*, "Mesenchymal stem cells transfer mitochondria to the cells with virtually no mitochondrial function but not with pathogenic mtDNA mutations," *PLoS One*, vol. 7, no. 3, Mar. 2012, doi: 10.1371/journal.pone.0032778.

[10] D. G. Phinney *et al.*, "Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs," *Nat. Commun.*, vol. 6, pp. 1–15, 2015, doi: 10.1038/ncomms9472.

[11] C. H. O. Davis *et al.*, "Transcellular degradation of axonal mitochondria," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 26, pp. 9633–9638, Jul. 2014, doi: 10.1073/pnas.1404651111.

[12] A. Nakajima, H. Kurihara, H. Yagita, K. Okumura, and H. Nakano, "Mitochondrial extrusion through the cytoplasmic vacuoles during cell death," *J. Biol. Chem.*, vol. 283, no. 35, pp. 24128–24135, Aug. 2008, doi: 10.1074/jbc.M802996200.

[13] M. A. Clark and J. W. Shay, "Mitochondrial transformation of mammalian cells," *Nature*, vol. 295, no. 5850, pp. 605–607, 1982, doi: 10.1038/295605a0.

[14] M. P. King and G. Attardi, "Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA," *Cell*, vol. 52, no. 6, pp. 811–819, Mar. 1988, doi: 10.1016/0092-8674(88)90423-0.

[15] C. A. Pinkert, M. H. Irwin, L. W. Johnson, and R. J. Moffatt, "Mitochondria transfer into mouse ova by microinjection," *Transgenic Res.*, vol. 6, no. 6, pp. 379–383, 1997, doi: 10.1023/A:1018431316831.

[16] Y. G. Yoon, C. L. Haug, and M. D. Koob, "Interspecies mitochondrial fusion between mouse and human mitochondria is rapid and efficient," *Mitochondrion*, vol. 7, no. 3, pp. 223–229, May 2007, doi: 10.1016/j.mito.2006.11.022.

[17] H. Margus, K. Padari, and M. Pooga, “Cell-penetrating peptides as versatile vehicles for oligonucleotide delivery,” *Molecular Therapy*, vol. 20, no. 3. Mol Ther, pp. 525–533, 2012, doi: 10.1038/mt.2011.284.

[18] A. Caicedo *et al.*, “MitoCeption as a new tool to assess the effects of mesenchymal stem/stromal cell mitochondria on cancer cell metabolism and function,” *Sci. Reports 2015 51*, vol. 5, no. 1, pp. 1–10, Mar. 2015, doi: 10.1038/srep09073.

[19] B. Shin *et al.*, “A Novel Biological Strategy for Myocardial Protection by Intracoronary Delivery of Mitochondria: Safety and Efficacy,” *JACC Basic to Transl. Sci.*, vol. 4, no. 8, pp. 871–888, Dec. 2019, doi: 10.1016/j.jacbts.2019.08.007.

[20] S. M. Emani, B. L. Piekarski, D. Harrild, P. J. del Nido, and J. D. McCully, “Autologous mitochondrial transplantation for dysfunction after ischemia-reperfusion injury,” *J. Thorac. Cardiovasc. Surg.*, vol. 154, no. 1, pp. 286–289, Jul. 2017, doi: 10.1016/j.jtcvs.2017.02.018.

[21] A. Masuzawa *et al.*, “Transplantation of autologously derived mitochondria protects the heart from ischemia-reperfusion injury,” *Am. J. Physiol. - Hear. Circ. Physiol.*, vol. 304, no. 7, pp. 966–982, 2013, doi: 10.1152/ajpheart.00883.2012.

[22] T. Kitani, D. Kami, S. Matoba, and S. Gojo, “Internalization of isolated functional mitochondria: Involvement of macropinocytosis,” *J. Cell. Mol. Med.*, vol. 18, no. 8, pp. 1694–1703, 2014, doi: 10.1111/jcmm.12316.

[23] E. E. Kesner, A. Saada-Reich, and H. Lorberboum-Galski, “Characteristics of Mitochondrial Transformation into Human Cells,” *Sci. Rep.*, vol. 6, May 2016, doi: 10.1038/srep26057.

[24] C. A. Pacak *et al.*, “Actin-dependent mitochondrial internalization in cardiomyocytes: Evidence for rescue of mitochondrial function,” *Biol. Open*, vol. 4, no. 5, pp. 622–626, 2015, doi: 10.1242/bio.201511478.

[25] H. C. Lin, S. Y. Liu, H. S. Lai, and I. R. Lai, “Isolated mitochondria infusion mitigates ischemia-reperfusion injury of the liver in rats,” *Shock*, vol. 39, no. 3, pp. 304–310, 2013, doi: 10.1097/SHK.0b013e318283035f.

3. *REVIEW*: "EXTRACELLULAR VESICLES, APOPTOTIC BODIES AND MITOCHONDRIA: STEM CELL BIOPRODUCTS FOR ORGAN REGENERATION"

Natalia Gebara & **Andrea Rossi** & Renata Skovronova, Justine Mariam Aziz, Amish Asthana, Benedetta Bussolati

Current Transplantation Reports (2020)

Current Transplantation Reports (2020) 7:105–113
https://doi.org/10.1007/s40472-020-00282-2

CELLULAR TRANSPLANTS (G ORLANDO, SECTION EDITOR)

Extracellular Vesicles, Apoptotic Bodies and Mitochondria: Stem Cell Bioproducts for Organ Regeneration

Natalia Gebara[1] · Andrea Rossi[1] · Renata Skovronova[1] · Justine Mariam Aziz[2] · Amish Asthana[2] · Benedetta Bussolati[1,3]

Published online: 27 April 2020

Abstract

Purpose of Review In the current work, we will present the characterization of the main different stem cell-derived vesicular bio-products with potential application in organ regeneration.

Recent Findings The therapeutic effects of stem cell therapy in organ repair, specifically those utilizing mesenchymal stromal cells, are largely dependent on the cells' release of different bio-products. Among these bio-products, extracellular vesicles (EVs) appear to play a major role due to their ability to carry and deliver bioactive material for modulation of cellular pathways in recipient cells. Concurrently, mitochondria transfer emerged as a new mechanism of cell communication, in which the bioenergetics of a damaged cell are restored. Finally, apoptotic bodies released by dying apoptotic stem cells contribute to stimulation of the tissue's stem cells and modulation of the immune response.

Summary Exploitation of isolated extracellular vesicles, mitochondria and apoptotic bodies in preclinical models of organ damage shows promising results. Here, we describe the results of the pre-clinical applications of stem cell vesicular products, as well as the first clinical trials approaching artificial administration of extracellular vesicles and mitochondria in human subjects and their possible benefits and limitations.

Keywords MSC · Regenerative medicine · MicroRNA · Exosomes · Microvesicles · Mitochondrial transfer · Apoptosis

Introduction

Organ failure is the most frequent cause of morbidity and mortality recorded in Europe and in the United States in recent decades. Organ dysfunction can be attributed to fibrosis, a pathological feature of many chronic inflammatory diseases, as its extensive remodelling of tissues leads to functional insufficiency [1]. The burden associated with fibrosis is disconcerting, representing in the United States almost half, and in the industrialized world about 30%, of all deaths attributed to fibrotic heart, lung, kidney and liver diseases [1, 2]. In addition, episodes of acute tissue injury, especially if severe and repeated, are closely associated to development of chronic organ disease [3].

It has therefore become of increasing interest in Regenerative Medicine to limit the progression of fibrosis, promote restoration of organ function in chronic settings and support organ repair after acute injury to regain tissue integrity. In this context, increasing studies underline the role of stem cell bio-products, including secreted soluble factors and extracellular vesicles (EVs), as powerful instruments in organ regeneration. EVs, in particular, have been proposed as a new form of intracellular messaging through their ability to reach distant organs and deliver the active cargo necessary for reprogramming of the target cells. In addition, EVs released by apoptotic cells, including apoptotic bodies (ApoBDs), are recently emerging as part of the therapeutic and immune-modulating mechanisms of injected stem cells within injured tissues [4] (Fig. 1).

Finally, stem cell therapy involves the transfer of mitochondria, the organelles responsible for cellular energy production, from stem cells to damaged cells. Mesenchymal stromal cells

Natalia Gebara, Andrea Rossi and Renata Skovronova contributed equally to this work.

This article is part of the Topical Collection on *Cellular Transplants*

✉ Benedetta Bussolati
benedetta.bussolati@unito.it

[1] Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy

[2] Wake Forest Univ. School of Medicine, Winston-Salem, NC, USA

[3] Molecular Biotechnology Centre, University of Torino, via Nizza 52, 10126 Torino, Italy

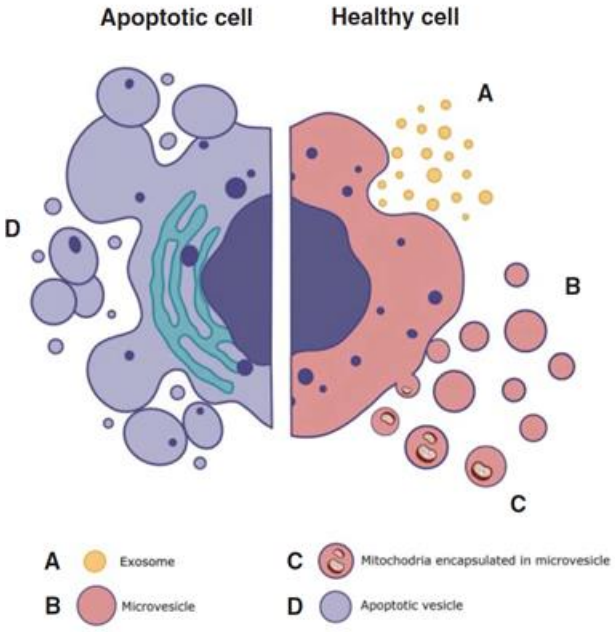


Fig. 1 Presentation of apoptotic and healthy cell secretome

(MSCs) are shown to transfer mitochondria to the recipient cells in different ways: encapsulated within EVs [5]; *via* cell-to-cell direct communication through tunnelling nanotubes; or through direct release of "naked" mitochondria into the extracellular microenvironment [6]. The organelle incorporates into the endogenous mitochondrial network of the damaged recipient cell that needs to be rescued, restoring its bioenergetic profile and health [7].

In this review, we will present the recent knowledge on mechanisms of action involved in the therapeutic effects of healthy and apoptotic EVs, as well as of mitochondria transfer, and the exploitation of these bio-products in preclinical models of organ damage. Finally, we will describe the first clinical trials approaching their use on human subjects and the possible benefits and limitations.

Extracellular Vesicles

Since the 'discovery' of EVs in blood plasma in 1946 by Erwin Chargaff and Rudolph West [8], interest in those cells-to-cell communicators has risen in almost all fields of biology and chemistry. EVs have been proven to naturally occur in prokaryotes, eukaryotes, plants and cells. EVs are membrane-bound, spherical particles enclosed in a lipid bilayer. In biological samples, EVs originate from their parental cell, taking up their internal and external composition. Guidelines from the International Society for Extracellular Vesicles (ISEV) classify three main categories of EVs; exosomes also named small EVs (~30–250 nm), large EVs or microvesicles (~100–1000 nm) and ApoBDs (>1 μm) [9••]. Small EVs and microvesicles are released from metabolically active cells, whereas ApoBDs are exclusively produced during cell apoptosis [10] (Fig. 1).

The content of an EV is dependent on its origin, size and the route of biogenesis. EV surface markers and cargo are specific to the three types of vesicles (Table 1) and are most commonly associated with the route of vesicle formation. The process of exosome formation begins with inward budding of early endosomes and formation of intraluminal vesicles; this involves the ESCORT complex, ALIX and tumour susceptibility gene 101 (TSG101), all of which are responsible for cargo sorting. Intraluminal vesicles mature into multivesicular bodies, followed by fusion with the cell membrane and release of vesicles into the extracellular environment. Alternatively, multi-vesicular bodies are degraded by lysosomes and their components recycled. Exosomes are distinguished by the presence of all three tetraspanin markers responsible for induction of membrane curvature (CD9, CD63 and CD81). Proteins that can be detected and are involved in exosome biogenesis include Rab, GTPases, annexin, flotillin,

Table 1 Composition, size and biogenesis route of EVs

Vesicle type	Origin	Size	Markers	Components
Exosomes	Endolysosomal pathway; fusion of MVB with cell membrane	30-250 nm	CD9, CD63, CD81 ESCORT components, TSG101, flotillin, Annexin	mRNA, miRNA and other non-coding RNAs; membrane and cytoplasmic proteins, lipids, receptors
Microvesicles	Cell membrane; bud off directly from cell surface	100-1000 nm	CD40	mRNA, miRNA and other non-coding RNAs; membrane and cytoplasmic proteins, lipids, receptors
Apoptotic bodies	Cell Membrane; membrane blebbing during apoptosis	>1μm	Phosphatidylserine, Calreticulin, Calnexin	Nuclear fragments and cell organelles

ALIX, TSG101, VPS4, heat shock protein (HSP70) and the ESCORT complex [11].

Microvesicles are formed by direct budding from the cell plasma membrane with involvement of cytoskeleton components and fusion machinery, though the process is not yet fully understood. Due to their biogenesis pathway, microvesicles are primarily composed of a plasma membrane and of cytosolic-associated proteins. Other commonly found components include heat shock proteins, integrins, post-translationally modified proteins and RNA species. Several structural components are shared between exosomes and microvesicles due to their similar release pathways and origin [11].

EVs have been designated as novel cell-to-cell communicators due to their effect on cells on a paracrine and endocrine level, specifically through the direct stimulation of cell surface receptors and transfer of bioactive molecules. Indeed, EV surface receptors may act as signalling complexes and directly stimulate target cells or, alternatively, transfer functionally active receptors from one cell to another. For instance, bystander B cells can acquire antigen receptors from activated B cells becoming specific activated antigen presenting cells for CD4 T cells [12].

In addition, the presence of a complex cargo (miRNA, RNA, proteins, lipids, cytokines and mitochondria) within EVs results in a multilevel modulation of cell functions in the recipient cells [13, 14]. Small RNA species, including miRNA, are present within EVs and, interestingly, recent studies found that the overall pattern of miRNA content of small and large EVs appears similar but distinctly different from that of the originating cells [15••], implying specific mechanisms of miRNA packaging into EVs. Moreover, extensive proteomic studies on EVs originating from cell cultures, tissue cultures and isolated bio-fluids have shown a significant EV protein content. Online databases created through the collaboration of EV research groups provide us with catalogued EV components, such as Exocarta, (www.exocarta.org), EVpedia (www.evpedia.info) and Vesiclepedia (www.microvesicles.org). EVs contain many common proteins involved in vesicle trafficking and serving as part of the cytoskeleton and the plasma membrane. Furthermore, specific protein content reflects the EV mechanism of generation and origin, as well as the cellular state of the EV originating cell. Finally, in addition to the structural functions of lipids in EV membranes, bioactive lipids such as eicosanoids, fatty acids and cholesterol are transferred by EVs to recipient cells. For instance, sphingomyelin has been shown to regulate angiogenesis in vitro and in vivo in tumour derived EVs [16].

Apoptotic EVs

Apoptosis is commonly known as programmed cell death. An apoptotic cell undergoes several morphological changes: membrane blebbing, membrane protrusion formation and generation of ApoBDs [17–19]. The membrane of ApoBDs reflects the main changes occurring in the cell surface of the apoptotic cell. In particular, apoptotic cells express markers promoting their removal by surrounding cells or macrophages before the cell membrane ruptures [20]. For instance, Calreticulin, an "eat me" ligand is physiologically silenced by the CD47 "don't eat me" ligand; and only expressed by cells and ApoBDs when CD47 is downregulated [21]. The size of ApoBDs ranges from 1 μm to 5 μm [22, 23]. This characteristic is similar to oncosomes (EVs secreted by cancer cells), but the biogenesis of these vesicles differs [24]. The number of ApoBDs produced per cell was quantified as 12.87 ± 3.23 per hour [25••]. In comparison, the average number of released EVs by mesenchymal stem cells was found be in the range of 2900 per cell, overnight [26].

During apoptosis, apoptotic microvesicles 0.1–1 μm in diameter and small exosome-like EVs are released [27, 28]. However, these vesicles are less characterized than the ApoBDs.

ApoBDs are characterized by the presence of externalized phosphatidylserine and by a permeable membrane. As mentioned above, they express phagocytosis-promoting signals, such as calreticulin [21] and calnexin [29]. In addition, ApoBDs express chemokines and adhesion molecules, such as CX3CL1/fractalkine and ICAM3, and MHC class II

molecules, allowing for direct antigen presentation to $CD4^+$ T cells and activation of immunological memory [30]. The cargo of ApoBDs consists of cellular components enclosed during protrusion. Due to this fact, the content of ApoEVs can be very diverse. Indeed, ApoBDs can contain microRNAs, RNA and DNA. Diversity of ApoBDs content affects their physiological properties. ApoBDs can be subdivided into two groups: DNA-carrying ApoBDs and cytoplasm-carrying ApoBDs. 5′ phosphorylated blunt-ended DNA can be used as a distinctive marker of DNA-carrying ApoBDs because it is exclusively found in ApoBDs, which undergo apoptosis and contain the DNA fragments [31].

Mitochondria

While mitochondria are widely considered the powerhouse of the cell, as they are responsible for ATP production through oxidative phosphorylation, these organelles are also involved in several other pathways. They serve a role in pluripotent stem cell maintenance [32], apoptosis and cell death regulation and proliferation capacity through complex interactions between p53 and reactive oxygen species (ROS) production [32]. Energy deprivation and mitochondria dysfunction has been strictly associated with end stage organ disease [33]. Therefore, while metabolic patterns and mitochondria contents can strictly vary among the organs, it appears evident that mitochondrial alterations are closely correlated with most of the clinical conditions that lead to organ failure [34, 35].

As mitochondria do not possess an efficient DNA-repair system [36], these organelles are typically recycled through mitophagy, a form of autophagy [37]. Moreover, the transfer of respiration-competent mitochondria from cell to cell emerged in the past few years [6] as a mechanism of damage repair or cell reprogramming [38]. The physiological mitochondria transfer is a biological phenomenon in which the organelle from a healthy donor cell is relocated into a stressed recipient cell, resulting in repair and survival of the damaged cell. During this process, the mitochondria's small size and plasticity allow it to be transported from donor to recipient cells through transporting mechanisms, such as tunneling nanotubes and microvesicles. The organelle is eventually incorporated into the endogenous mitochondrial network of the recipient cell that needs to be rescued, restoring the cell to its bio-energetic profile and health [39].

The incorporation of respiration-competent mitochondria within released microvesicles has been extensively studied in MSCs, where, once internalized, mitochondria-containing microvesicles can rescue cells from injury or act as reprogramming factors [5, 40••]. In 2012, Islam et al. demonstrated that mitochondria can be transferred in vivo through a mouse model of acute lung injury [38]. Firstly, mitochondria-labelled MSCs were administered by injection into the damaged lungs. These MSCs homed in the damaged tissue and produced microvesicles containing the labelled mitochondria 4 h post-injection. The microvesicles were then directly transferred into the damage lung cells, resulting in the restoration of their ATP concentrations and secretory responses [38].

PMT has also been observed through the establishment of tunnelling nanotubes (TNTs) [6]. TNTs are filamentous connections formed by protrusions of a cell's membrane and are used to share organelles and contents of the cell's cytoplasm with other cells [41]. The utilization of TNTs in PMT has been observed in transfers between MSCs and macrophages, which served to enhance macrocytosis and activate antimicrobial response [42]. Moreover, Sinclair et al. demonstrated that the transfer of mitochondria via TNTs is essential due to the regenerative capacity exerted by MSCs [41]. However, the role and mechanism of PMT through the establishment of TNTs is poorly understood. The last and least known mechanism of PMT is the direct release of mitochondria in the extracellular microenvironment, usually in response to cell stress. "Naked" mitochondria can be encapsulated in a vacuole and then extruded by apoptotic cells, specifically hepatocytes [43]. Similarly, platelets can release respiration-competent mitochondria as a mediator of innate immune response [44].

Therapeutic Effects of EVs, ApoBDs and Mitochondria for Organ Regeneration

EV Reprogramming of Injured Tissue

Following the emerging interest of stem cell therapy, an increasing number of research and pharmaceutical groups are focusing on stem cell derived EVs, specifically MSC-derived EVs, as a new form of therapeutic agents for organ regeneration and protection [45–48]. Due to the ability of MSC-derived EVs to transfer therapeutic molecules, such as mRNAs, miRNAs and protein, and several of their regenerating effects to MSCs, this source of EVs is one of the most studied in Regenerative Medicine. Moreover, MSCs produce a higher number of EVs in comparison with other stem cells [49]. MSC-derived EVs were proven to modulate the immune system and stimulate regeneration in a multitude of preclinical models, including graft-versus-host-disease, lung, liver, kidney and cardiovascular injury [45]. There are a significant number of studies describing the pro-regenerative effects of stem cell-EVs for organ regeneration, but it is not within the scope of this review to detail all the different preclinical models of application.

As detailed above, the therapeutic effect of stem cell-EVs on organ repair is related to transfer of pro-regenerative proteins or microRNAs. For instance, although numerous factors have demonstrated the therapeutic effects of different EV models and origins, not a single agent emerged as pivotal or

indispensable. Therefore, it can be hypothesized that not a single factor, but rather a synergic and multi-target action of EV components is responsible for the therapeutic EV results. Indeed, the common and characterizing action of therapeutic MSC-EVs can be described as a reprogramming activity on tissue expression profiles. For instance, in models of kidney and liver injury [26], the expression profile of the EV-treated diseased organ, as assessed by RNA sequencing, correlated with that of the normal tissue. Moreover, in models of chronic tissue injury, an upregulation of anti-fibrotic genes and downregulation of pro-fibrotic genes was common to the different diseases as well as stem cell sources [50, 51]. Through modulation of their phenotype and subsequent secretomes, therapeutic utilization of EVs may benefit from an in vitro stimulation or manipulation of the generating cell source. For instance, ischemic or hormonal stimulation may ameliorate EV activities [52–54]. In addition, EV administration for chronic diseases might require multiple administrations. The possible development of immune reactions in this setting has not yet been studied in depth.

Regenerative Effect of Apoptotic Body Phagocytosis

Due to the rapid clearance of damaged cells by immune cells, namely phagocytes, ApoBDs play a major role in immune regulation [30, 55]. ApoBDs are emerging as a pivotal tool in cell-to-cell communication between damaged and healthy cells, therefore modulating mechanisms of organ repair. Indeed, ApoBDs may stimulate proliferation of resident stem/progenitor cells, improving tissue regeneration and replacing damaged cells [25••, 56]. For instance, phagocytosis of the ApoBDs by hepatic stellate cells can promote their differentiation and increase their cell survival [57]. Moreover, ApoBDs' engulfment may support MSC homeostasis. In particular, systemic infusion of exogenous ApoBDs was able to rescue apoptotic MSCs by transferring RNF146 and miR-328-3p and activating the Wnt/β-catenin signalling [58•]. In parallel, in zebrafish, dying epithelial stromal cells of the epidermis were observed to generate Wnt8a enriched ApoBDs, supporting the hypothesis of ApoBDs being biologically active vehicles in cell-to-cell communication [25••]. Neighbouring p63-positive stromal cells engulfed the ApoBDs, which caspase-dependently activated Wnt signalling and stimulated cell proliferation and tissue homeostasis over 24 h. In this model, inhibition of apoptosis significantly reduced the number of proliferating stromal cells. On the contrary, overexpression of the Wnt pathway in combination with apoptosis induced a significant increase of stromal cell proliferation [25••].

ApoBDs can deliver microRNAs, DNA and other genetic material to target cells, resulting in a multitude of different effects. For example, miRNA-126, present in endothelial ApoBDs, promoted chemokine CXCL12 expression in healthy endothelium, and repeated administration of those ApoBDs in mice with atherosclerosis induced an atheroprotective effect [46].

Although the use of ApoBDs generated in culture as therapeutic has not yet been tested, their role appears of increasing interest in the field of Regenerative Medicine.

Artificial Mitochondria Transfer

In recent years, artificial mitochondrial transfer (AMT) emerged as a new possible therapeutic option for tissue repair. AMT has been intensively investigated in cardiac disease models. In a rabbit model of cardiac IRI, the injection of viable respiration-competent mitochondria, isolated from donor rabbit cardiac or muscular tissues, was able to significantly reduce the infarct size area, kinase MB, cardiac troponin-I and apoptosis in the regional ischemic zone [47, 48]. AMT has also been recently tested in a mouse model of heterotopic heart transplantation: mitochondria isolated from gastrocnemius muscle were autologously administrated in the heart coronary ostium before and after the transplant. Within 24 h after transplant, necrosis and neutrophil infiltration were significantly decreased compared with the vehicle-treated group. Moreover, the mitochondrial treatment significantly enhanced the beating score after transplant [59]. Interestingly, these papers demonstrated both in vitro and in vivo that fully differentiated cells can be used as a source for the mitochondrial injection.

Moreover, transfer of mitochondria, mainly derived from MSCs, has been proven effective in other pathologic models involving liver, brain and kidney. In a rat model of liver IRI, the intra-splenic administration of MSC mitochondria mitigated the necrosis of hepatocytes as well as reduced the expression of mitochondrial-induced apoptosis markers [60]. In the kidney, the effectiveness of AMT was demonstrated by rescue of damaged renal proximal tubular cells. In vitro, the administration of MSC-derived mitochondria reduced ROS production and increased the expression of the tubular marker megalin and mitochondrial superoxide dismutase 2, whereas in vivo, both the tubular basement membrane and brush border were protected [61]. Moreover, in normal mice, the administration of mitochondria improved endurance during forced swimming test. Finally, AMT has also been used in vivo in a murine model of Parkinson's disease and in vitro for the regeneration of damaged hippocampal cells [62, 63].

Although of great novelty, these therapeutic approaches have already started to be applied in the human setting.

Clinical Trials Involving EVs and Mitochondria Transfer

All the clinical trials concerning MSC-EVs and ATM can be found at www.clinicaltrails.gov. Although the majority of listed trials focus on the diagnostic properties of EVs, there

are five trials testing the therapeutic applications of MSCs-EVs and two proposing the use of ATM (Table 2).

A first trial is designed to test the anti-inflammatory properties of umbilical cord derived MSCs-EVs to prevent the destruction of pancreatic β-cell islets. The MSCs-EVs will be administered intravenously in two doses, the first dose of exosomes and, after seven days, the second dose of microvesicles (NCT02138331). Two other clinical trials using EVs will involve allogeneic MSCs-EVs. One of them will administer EVs enriched by miR-124 for the treatment of acute ischemic stroke (NCT03384433). The second clinical trial will attempt to treat lesions in patients affected by dystrophic epidermolysis bullosa (NCT04173650). The last clinical trial using EVs that is in the recruiting phase focuses on promoting the healing and recovery of refractory macular holes through direct injection of MSC exosomes to the site of the injury (NCT03437759). Finally, the only concluded trail to date used umbilical cord derived MSCs-EVs to inhibit the progression of chronic kidney disease in patients with grade III-IV CKD [64]. The study showed stabilization of the disease progression, as confirmed by stable levels of glomerular filtration rate, serum creatinine and blood urea in treated patients, and an increased level of anti-inflammatory factors (TGF-β1 and IL-10) in comparison with the matching placebo group.

The first clinical trial using administration of isolated mitochondria for the treatment of myocardial IRI has also been concluded with positive results [65]. Mitochondria were isolated from non-ischemic skeletal muscles and injected in the myocardium of paediatric patients with myocardial IRI. No adverse effects were detected after AMT, and four out of five patients demonstrated an enhancement in ventricular function [65].

Other clinical trials using AMT are focused on the improvement of infertility treatments (Table 2). Through autologous micro-injection of mitochondria prior to intracytoplasmic sperm injection, the patients' oocyte quality was enhanced. In the first trial, concluded in 2017, mitochondria were isolated from autologous ovarian stem cells and directly injected into the oocytes themselves. To date, no results have been published. Embryo quality has been quantified through the pregnancy rate after treatment and morphological evaluation of the treated embryos. In the second clinical trial, which is still ongoing, mitochondria will be isolated from autologous bone marrow-MSCs and administrated immediately before intra-cytoplasmic sperm injection in the oocytes. Live birth

Table 2 Current clinical trials concerning the use of EVs or AMT as therapeutic agents

	Intervention	N. pts	Follow up	State	Location	Number/Ref.
Diabetes Mellitus type 1	Two doses of MSC-EVs	20	3 months	Unknown	Sahel Teaching Hospital Sahel, Cairo, Egypt	NCT02138331
Chronic kidney disease	Two doses of umbilical cord MSC-EVs (100 μg/kg/dose)	20	1 year	Concluded	Sahel Teaching Hospital Sahel, Cairo, Egypt	Nassar et al.
Molecular degeneration	20–50 mg of cord tissue MSC-EVs injected directly around macular hole	44	24 weeks	Recruiting	Tianjin Medical University Hospital Tianjin, China	NCT03437759
Cerebrovascular disorders acute ischemic stroke	Allogeneic MSC-EVs enriched by miR-124	5	12 months	Not recruiting yet	Shahid Beheshti University of Medical Sciences, Tehran, Iran	NCT03384433
Dystrophic Epidermolysis Bullosa	Allogeneic MSC-EVs applied directly to lesions, blisters for 60 days	30	2 months	Not recruiting yet	Aegle Therapeutics, Miami, Florida USA	NCT04173650
Repetition failure	Clinical Application of Autologous Mitochondria Transfer for Improving Oocyte Quality.	50	2-3 years	Not recruiting yet		NCT03639506
Infertility	Amelioration of oocyte quality using autologous mitochondria transfer	59	5 months	Concluded	Valencia, Spain	NCT02586298
IRI	Autologous mitochondrial transfer for dysfunction after ischemia-reperfusion injury	5	6 days	Concluded	Boston, MA, USA	Emani et al.

rate, pregnancy rate, number of oocytes retrieved and fertility rate are going to be evaluated.

Conclusion

Numerous discoveries within the Regenerative Medicine field have highlighted the bioactivity of stem cell bio-products and their role in cell-to-cell communication. In particular, EVs are the most advanced as potential therapeutic agents due to their ability to modulate the function of targeted cells. Together with stem cell-EVs, proven to be of therapeutic effect in a large variety of pre-clinical models, ApoBDs and AMT can be utilized for selected and specific applications.

The major issue with use of EVs in clinical practice is the standardization of EVs isolation, usage and storage. However, once those issues can be overcome, using EVs as therapeutic agents provides solutions to numerous complications caused by stem-cell therapy, including immune compatibility, maldifferentiation and tumourigenicity. EV therapy allows for dosage control, evaluation of safety and potency equivalent to pharmaceutical agents. In comparison to stem cell therapy, EVs are potentially an easier option as they can be directly obtained from the medium of cultured cells, massively produced and stored without the application of toxic cryopreservative agents and/or loss of EV potency [66]. The biological properties of EV allow for modification of the EV content to obtain desired cell-specific effects. Encapsulation of effector molecules (nucleic acids, lipids and proteins) by EVs allows delivery of its cargo without the risk of degradation. Genetically modified EVs may offer a more effective and natural solution than usage of FDA-approved lipid nanoparticles, which pose a low-dose toxicity upon cell entry [67].

The application of ApoBDs is still poorly explored, as several aspects still require further investigation. For instance, apoptotic vesicles are quite heterogeneous and may have different compositions and properties depending on their size. While EVs released by primary murine aortic endothelial apoptotic cells enhanced inflammation in mice transplanted with an MHC-incompatible graft, ApoBDs did not show this behaviour [28]. Therefore, as increasing experimental evidence suggests, the therapeutic effects of stem cells are due to their clearance by the immune system [4, 68]. For this reason, therapeutic utilization of these cell products appears of interest.

Finally, the use of MSCs as a source of mitochondria for AMT is a novel, therapeutic option that shows regenerative effects in the treatment of acute cell damage. However, more studies are required for better understanding of mitochondria internalization, their fate once inside the injured cells and how mitochondria can survive in the extracellular microenvironment or the blood flow during AMT.

Acknowledgements We would like to thank Lola Buono for her contribution to the review by designing Figure 1.

Funding This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreements No. 813839 and 765274.

Compliance with Ethical Standards

Conflict of Interest All authors declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

1 Roesnbloom J, Castro SV, Jimenez SA. Review narrative review: fibrotic diseases: cellular and molecular mechanisms and novel therapies. Ann Intern Med. 2010;152(3):159–66.
2 Jha V, et al. Chronic kidney disease: Global dimension and perspectives. Lancet. 2013;382(9888):260–72.
3 Chawla LS, Eggers PW, Star RA, Kimmel PL. Acute kidney injury and chronic kidney disease as interconnected syndromes. N Engl J Med. 2014;371(1):58–66.
4 Galleu A, et al. Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. Sci Transl Med. 2017;9:416.
5 Islam MN, et al. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nat Med. 2012;18(5):759–65.
6 Caicedo A, Aponte PM, Cabrera F, Hidalgo C, Khoury M. Artificial mitochondria transfer: current challenges, advances, and future applications. Stem Cells Int. 2017;2017:7610414.
7 Murray LMA, Krasnodembskaya AD. Concise review: intercellular communication via organelle transfer in the biology and therapeutic applications of stem cells. Stem Cells. 2019;37(1):14–25.
8 Chargaff E, West R. The biological significance of the thromboplastic protein of blood. 1946;166(1):189–97.
9•• Théry C, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014

guidelines. J Extracell Vesicles. 2018;7(1). **The paper provides a throughout description of minimal information needed to work with extracellular vesicles as well as their chracterisation and description**.
10 Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): Exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol. 2013;113(1):1–11.
11 Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. 2015;1:1–60.
12 Quah BJC, Barlow VP, McPhun V, Matthaei KI, Hulett MD, Parish CR. Bystander B cells rapidly acquire antigen receptors from activated B cells by membrane transfer. Proc Natl Acad Sci U S A. 2008;105(11):4259–64.
13 Chai R, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res. 2010;4(3):214–22.
14 Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics. 2010;73(10):1907–20.
15•• Jeppesen DK, et al. Reassessment of Exosome Composition. Cell. 2019;177(2):428–445.e18. **The recent study shows the composisiton of exosomes to be different than prviously thought, thus changing the ideas behing exosomes and their potential applications**.
16 Kim CW, Lee HM, Lee TH, Kang C, Kleinman HK, Gho YS. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res. 2002;62(21):6312–7.
17 Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. Br J Cancer. 1972;26:239–57.
18 Atkin-Smith GK, et al. A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. Nat Commun. 2015;6:7439.
19 Alberts B, Bray D, Alexander J, Lewis J. *Základy buněčné biologie*. Espero: Partizánske; 2006.
20 Depraetere V. 'Eat me' signals of apoptotic bodies. Nat Cell Biol. 2000;2(6):2000.
21 Gardai SJ, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005;123(2):321–34.
22 Hristov M, Erl W, Linder S, Weber PC. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. Blood. 2004;104(9):2761–6.
23 Tixeira R, et al. Defining the morphologic features and products of cell disassembly during apoptosis. Apoptosis. 2017;22(3):475–7.
24 Jiang L, et al. Determining the contents and cell origins of apoptotic bodies by flow cytometry. Sci Rep. 2017;7(1):1–12.
25•• Brock CK, et al. Stem cell proliferation is induced by apoptotic bodies from dying cells during epithelial tissue maintenance. Nat Commun. 2019;10(1):1–11. **This recent study shows that ApoBDs stimulates proliferation of stem cells improving tissue regeneration**.
26 Collino F, et al. AKI recovery induced by mesenchymal stromal cell-derived extracellular vesicles carrying microRNAs. J Am Soc Nephrol. 2015;26(10):2349–60.
27 Karpman D, Ståhl AL, Arvidsson I. Extracellular vesicles in renal disease. Nat Rev Nephrol. 2017;13(9):545–62.
28 Dieudé M, et al. The 20S proteasome core, active within apoptotic exosome-like vesicles, induces autoantibody production and accelerates rejection. Sci Transl Med. 2015;7(318):1–18.
29 Lunavat TR, et al. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells – evidence of unique microRNA cargos. RNA Biol. 2015;12(8):810–23.
30 Caruso S, Poon IKH. Apoptotic cell-derived extracellular vesicles: More than just debris. Front Immunol. 2018;9:1486.
31 Hauser P, Wang S, Didenko VV. Apoptotic Bodies: Selective Detection in Extracellular Vesicles. Methods Mol Biol. 2017;1554:127–41.
32 Folmes CDL, et al. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metab. 2011;14(2):264–71.
33 Triolo F, Gridelli B. End-stage organ failure: Will regenerative medicine keep its promise? Cell Transplant. 2006;15(SUPPL. 1):S3–10.
34 Červinka M. The role of mitochondria in apoptosis induced in vitro. Gen Physiol Biophys. 1999;18:33–40.
35 Jassem W, Heaton ND. The role of mitochondria in ischemia/reperfusion injury in organ transplantation. Kidney Int. 2004;66(2):514–7.
36 Zinovkina LA. Mechanisms of mitochondrial DNA repair in mammals. Biochem. 2018;83(3):233–49.
37 Ding WX, Yin XM. Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem. 2012;393(7):547–64.
38 Sinha P, Islam MN, Bhattacharya S, Bhattacharya J. Intercellular mitochondrial transfer: bioenergetic crosstalk between cells. Curr Opin Genet Dev. 2016;38(June):97–101.
39 Pacak CA, et al. Actin-dependent mitochondrial internalization in cardiomyocytes: Evidence for rescue of mitochondrial function. Biol Open. 2015;4(5):622–6.
40•• Phinney DG, et al. Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs. Nat Commun. 2015;6:8472. **In this study phinney et al. shed light on the complex mechanism that allow macrophages to engulf mitochondria from MSCs, in order to increase their bioenergetics after oxidative stress increase**.
41 Sinclair KA, Yerkovich ST, Hopkins PMA, Chambers DC. Characterization of intercellular communication and mitochondrial donation by mesenchymal stromal cells derived from the human lung. Stem Cell Res Ther. 2016;7(1):1–10.
42 Jackson MV, et al. Mitochondrial transfer via tunneling nanotubes is an important mechanism by which mesenchymal stem cells enhance macrophage phagocytosis in the in vitro and in vivo models of ARDS. Stem Cells. 2016;34(8):2210–23.
43 Nakajima A, Kurihara H, Yagita H, Okumura K, Nakano H. Mitochondrial extrusion through the cytoplasmic vacuoles during cell death. J Biol Chem. 2008;283(35):24128–35.
44 Boudreau LH, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase a to promote inflammation. Blood. 2014;124(14):2173–83.
45 Mendt M, Rezvani K, Shpall E. Mesenchymal stem cell-derived exosomes for clinical use. Bone Marrow Transplant. 2019;54:789–92.
46 Zernecke A, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci. Signal. 2009;2(100):1–13.
47 Shi X, Zhao M, Fu C, Fu A. Intravenous administration of mitochondria for treating experimental Parkinson's disease. Mitochondrion. 2017;34:91–100.
48 Masuzawa A, et al. Transplantation of autologously derived mitochondria protects the heart from ischemia-reperfusion injury. Am. J Physiol Hear Circ Physiol. 2013;304(7):966–82.
49 Gatti S, et al. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. Nephrol Dial Transplant. 2011;26(5):1474–83.
50 Bruno S, et al. HLSC-derived extracellular vesicles attenuate liver fibrosis and inflammation in a murine model of non-alcoholic steatohepatitis. Mol Ther. 2020;28(2):1–11.
51 Grange C, Skovronova R, Marabese F, Bussolati B. Stem cell-derived extracellular vesicles and kidney regeneration. Cells. 2019;8(10):1240.

52 Zhu J, et al. Myocardial reparative functions of exosomes from mesenchymal stem cells are enhanced by hypoxia treatment of the cells via transferring microRNA-210 in an nSMase2-dependent way. Artif Cells Nanomed Biotechnol. 2018;46(8):1659–70.

53 Lopatina T, Bruno S, Tetta C, Kalinina N, Porta M, Camussi G. Platelet-derived growth factor regulates the secretion of extracellular vesicles by adipose mesenchymal stem cells and enhances their angiogenic potential. Cell Commun Signal. 2014;12(1):1–12.

54 Zanjani ED, Banisadre M. Hormonal stimulation of erythropoietin production and erythropoiesis in anephric sheep fetuses. J Clin Invest. 1979;64(5):1181–7.

55 Caruso S, et al. Defining the role of cytoskeletal components in the formation of apoptopodia and apoptotic bodies during apoptosis. Apoptosis. 2019;24(11–12):862–77.

56 Li B, et al. Bone marrow mesenchymal stem cells protect alveolar macrophages from lipopolysaccharide-induced apoptosis partially by inhibiting the Wnt/β-catenin pathway. Cell Biol Int. 2015;39(2):192–200.

57 Jiang JX, Mikami K, Venugopal S, Li Y, Török NJ. Apoptotic body engulfment by hepatic stellate cells promotes their survival by the JAK/STAT and Akt/NF-κB-dependent pathways. J Hepatol. 2009;51(1):139–48.

58• Liu D, et al. Circulating apoptotic bodies maintain mesenchymal stem cell homeostasis and ameliorate osteopenia via transferring multiple cellular factors. Cell Res. 2018;28(9):918–33. **The paper shows ApoBDs activates Wnt/β-catenin signalling which can ameliorate MSCs in apoptosis**.

59 Moskowitzova K, et al. Mitochondrial transplantation prolongs cold ischemia time in murine heart transplantation. J HearT Lung Transplant. 2019;38(1):92–9.

60 Lin HC, Liu SY, Lai HS, Lai IR. Isolated mitochondria infusion mitigates ischemia-reperfusion injury of the liver in rats. Shock. 2013;39(3):304–10.

61 Konari N, Nagaishi K, Kikuchi S, Fujimiya M. Mitochondria transfer from mesenchymal stem cells structurally and functionally repairs renal proximal tubular epithelial cells in diabetic nephropathy in vivo. Sci Rep. 2019;9(1):1–14.

62 Chien L, Liang MZ, Chang CY, Wang C, Chen L. Mitochondrial therapy promotes regeneration of injured hippocampal neurons. Biochim Biophys Acta - Mol Basis Dis. 2018;1864(9):3001–12.

63 Shi X, Zhao M, Fu C, Fu A. Intravenous administration of mitochondria for treating experimental Parkinson's disease. Mitochondrion. 2017;34:91–100.

64 Nassar W, et al. Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases. Biomater Res. 2016;20(21):1–11.

65 Emani SM, Piekarski BL, Harrild D, del Nido PJ, McCully JD. Autologous mitochondrial transplantation for dysfunction after ischemia-reperfusion injury. J Thorac Cardiovasc Surg. 2017;154(1):286–9.

66 Ohno SI, Drummen GPC, Kuroda M. Focus on extracellular vesicles: Development of extracellular vesicle-based therapeutic systems. Int J Mol Sci. 2016;17(2):172.

67 Murphy DE, et al. Extracellular vesicle-based therapeutics : natural versus engineered targeting and trafficking. Exp Mol Med. 2019;51:1–12.

68 Vagnozzi RJ, et al. An acute immune response underlies the benefit of cardiac stem-cell therapy. Nature. 2019;577:405–9.

4. MECHANISMS AND STRATEGIES FOR KIDNEY PRESERVATION

General structure and functions of the kidney

The Kidneys are two organs located on the left and right in the retroperitoneal space, high in the abdominal cavity, one on each side of the spine, and lie in a retroperitoneal position at a slightly oblique angle. Blood is received from the renal arteries and exits into the renal veins. Both the kidneys are attached to a ureter, that carries extracted urine to the bladder. The functional part of the kidney is divided into two major structures: the cortex and the medulla (fig. 8). These two structures together form a series of cone shaped renal lobes, each containing a cortex surrounded by a portion of medulla called renal pyramid. Between the pyramids, there are the projections of cortex called renal columns. The urine-producing functional structures of the kidney are the nephrons, that span the cortex and the medulla. The renal corpuscle, the initial filtering portion of the nephron, is located in the cortex; whereas the second part, the renal tubule, passes through the cortex to arrive in the medulla (fig. 8). The tip of each renal pyramid, called papilla, empties the created urine into a minor calyx; minor calyces empties into a major calix. Major calices empties into the renal pelvis, which becomes the ureter. All these structures are surrounded by hilar fat and lymphatic tissues [1].

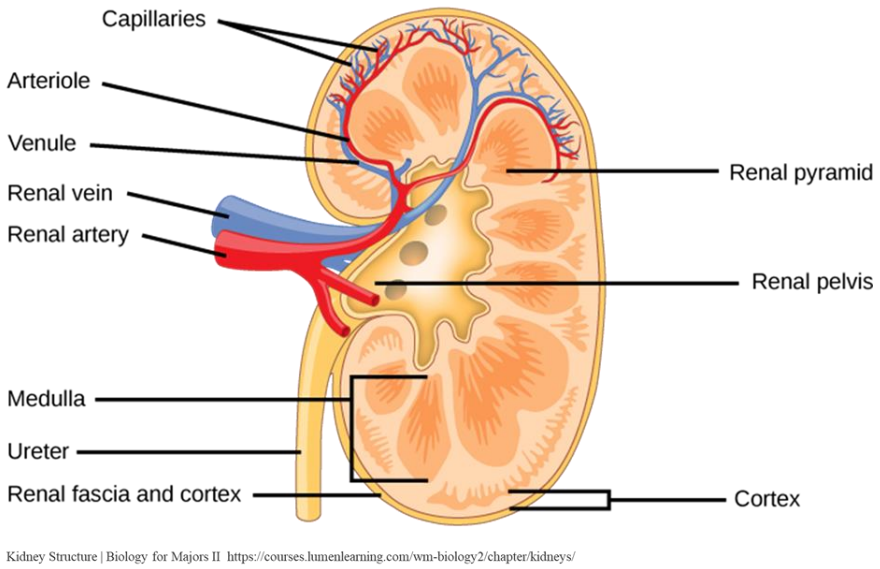


Figure 8. The internal structure of the kidney.

The main function of the kidney is to excrete a variety of waste products generated during the metabolism and accumulated in the blood stream. The purification passages, formed by filtration, reabsorption, secretion, and excretion, occurred in the nephron. The first passage is the filtration phase, which takes place in the renal corpuscle. Cells and large proteins are retained while materials of smaller molecular weights are filtered from the blood to make an ultrafiltrate that eventually becomes urine. The second step is reabsorption, where some molecules are transported back from the ultrafiltrate to the peritubular capillary. During this phase, glucose, amino acids, sodium, calcium, and other molecules are reabsorbed. Nearly 55% of water present in the ultrafiltrate is reabsorbed. During the secretion other molecules are

transported from the peritubular capillary through the interstitial fluid, then through the renal tubular cell and into the ultrafiltrate. The last step is the excretion. The ultrafiltrate passes out of the nephron and travels through the collecting duct, which is part of the collecting duct system, and then to the ureters where it is renamed urine. In addition to transporting the ultrafiltrate, the collecting duct also takes part in reabsorption.

The other functions of the kidney includes:

- The secretion of a variety of hormones, such as erythropoietin, calcitriol, and renin. Erythropoietin, in particular, is released in response to hypoxia in the renal circulation, stimulating erythropoiesis.

- The long-term regulation of blood pressure depends upon kidney, mainly through the renin–angiotensin system. When renin levels are elevated, the concentrations of angiotensin II and aldosterone increase, leading to increased sodium chloride reabsorption, expansion of the extracellular fluid compartment, and an increase in blood pressure. Conversely, when renin levels are low, angiotensin II and aldosterone levels decrease, contracting the extracellular fluid compartment, and decreasing blood pressure.

- Together with lungs, the kidneys maintain acid–base homeostasis, which is the maintenance of pH around a relatively stable value. While the lungs the lungs contribute to acid–base homeostasis by regulating CO_2 concentration,

the kidneys reabsorb and regenerate bicarbonate from urine, and to excrete hydrogen ions and fixed acids into urine.

- The kidneys are able to regulate the plasma osmolarity through the antidiuretic hormone (ADH) secreted by the posterior pituitary gland. An increase in osmolality causes the gland to secrete ADH, resulting in water reabsorption by the kidney and an increase in urine concentration [1].

Clinical conditions and loss of kidney function

The best available indicator of overall kidney function is glomerular filtration rate (GFR), which equals the total amount of fluid filtered through all the functioning nephrons per unit of time. Interestingly, the kidney can maintain a physiological level of GFR, despite progressive destruction of nephrons, because the remaining normal nephrons develop hyperfiltration and compensatory hypertrophy. For this reason, the decline of kidney function is gradual and initially may present asymptomatically. Although hyperfiltration and hypertrophy of residual nephrons are beneficial for the maintenance of GFR, it is found to be a major cause of progressive renal dysfunction. The increased glomerular capillary pressure may damage the capillaries, leading to focal and segmental glomerulosclerosis (FSGS) and eventually to global glomerulosclerosis [2]. The prolonged loss of function of the kidneys (expressed in a reduced GFR) for more than three months is defined as chronic kidney disease (CKD) [3]. When GFR is less than 15 mL/min per 1·73 m^2 (the GFR for CKD is 60 mL/min per 1·73 m^2), a person has reached end stage

kidney disease (ESKD), at which point kidney function is no longer able to sustain life over the long term. The prevalence of ESKD is continuously rising, reaching 746,557 in 2017 (versus 727,912 in 2016) only in the USA. The total US Medicare spending on both CKD and ESKD patients was in excess of $120 billion in 2017 [4]. Options for patients with ESKD are kidney replacement therapy (in the form of dialysis or kidney transplantation), or conservative care (also called palliation or non-dialytic care) [5]. The disorder is associated with numerous hospitalizations, increased healthcare costs, and numerous metabolic changes. The mortality rates for patients with end-stage renal disease are significantly higher than those without the disease. Even with timely dialysis, the death rates vary from 20% to 50% over 24 months [6].

Transplantation is generally the treatment of choice for those suffering from ESRD. Not only transplantation offer improved quality of life and increased longevity relative to dialysis, but it also reduces end-stage renal disease program expenditures, providing savings to Medicare. Unfortunately, the amount of transplantable organs is continuously growing, while the organ supply has reached a plateau. For this reason, in the last years, the conditions and circumstances in organ donation radically changed, with a significant boost in donor age and donation from non-heart-beating donors. In the most developed countries, donation after cardiac death (DCD) and extended criteria donor (ECD) organs make up more than 50% of the total organ pool. The ECD criteria most widely used in kidney transplantation are the OPTN-approved criteria, as described by Metzger et al. [7]. The most important parameters to be considered are age, weight, height, population,

history of hypertension, history of diabetes, cause of death, hepatitis C status, serum creatinine, and DCD. The most important burden with the ECD is the IRI. IRI is a period of sudden disrupted blood flow to the organ followed by the restoration of arterial perfusion. The ischemic period leads to a dramatic decrease in energy supply, which causes widespread damage to the cell and thus of the organ. The absence of oxygen and nutrient leads to ATP depletion, and pH increasing, with the subsequent intracellular Ca^{2+} overload and increase in lactate production. After the ischemic period, reperfusion is characterized by a dynamic process of cytokine production, neutrophil activation, and ROS release that causes endothelial and epithelial damage, widespread inflammation, and disruption of parenchymal architecture [8]. Intracellularly, IRI is followed by the complete disruption of the mitochondrial network. This generates apoptosis and

necrosis in the tissue and then in the whole organ (fig. 9).

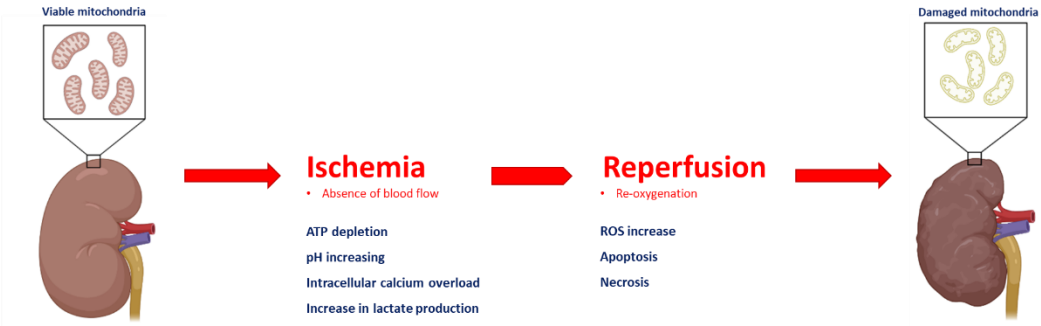


Figure 9. Graphical representation of IRI. Ischemia generate ATP depletion, pH increasing, intracellular calcium overload and increase in lactate production. The subsequent reperfusion phase led to ROS increase, apoptosis and, eventually, necrosis.

With the pressure to maintain and further improve the excellent short- and long-term results, but working with very different resources, the most prominent immediate challenge is to better define and preserve, if not improve, organ quality in transplantation.

Ischemia Reperfusion Injury and Its Role in Kidney Transplantation, Delayed Graft Function

As mentioned, tubular epithelial cells are particularly susceptible to the reduced oxygen supply caused by the ischemic phase of IRI. Anaerobic glycolysis and ATP degradation form superoxide radicals and an acidic milieu, together with the subsequent restoration of circulation results in inflammation and production of ROS. All these factors bring mitochondria damage and thus to the activation of the apoptotic pathway in tubular epithelial cells. The amount of damage, that is usually proportional to the duration of the ischemic period, may eventually lead to delayed graft function (DGF) [9]. Even if recently challenged, the traditional definition of DGF is based on the requirement of dialysis during the first post-operative week. Other classifications are based on creatinine reduction ratio and 24-h creatinine excretion from post-transplant Days 1–2 [10]. Other than the definitions, DGF results in a dialysis period, usually combined prolonged hospitalization, increase in the complexity of the therapeutic approach and an enhanced probability of infections. All these effects can dramatically alter the outcome of transplantation. In fact, it has been reported that DGF reduced the 5-year graft survival rate by 10– 15% and shortened the half-life by about 2 years [10]. The presence of kidney fibrosis is

strictly related to the outcome of the graft and to DGF. The presence of AKI or Epithelial-to-mesenchymal transition (EMT) in injured epithelial cells can both lead to this condition in kidney graft.

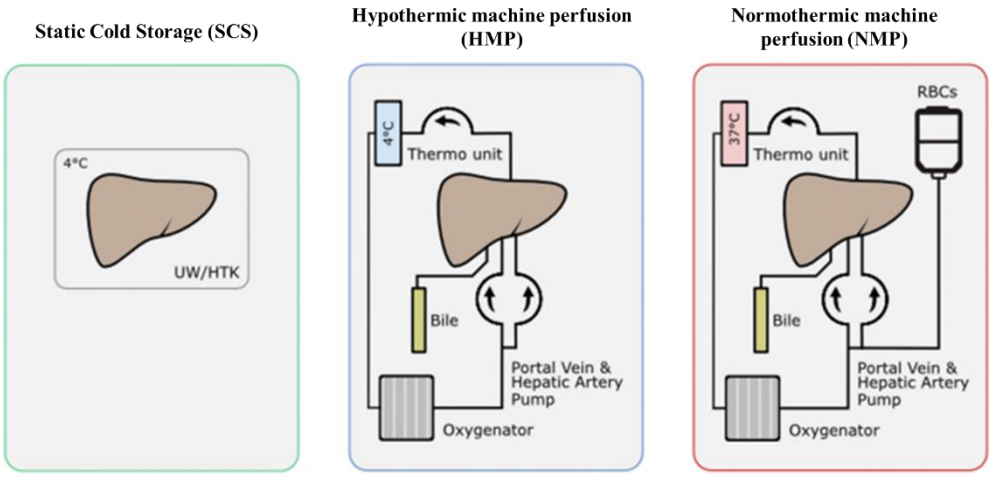
There are multiple risk factors associated with the insurgence of DGF. The most important donor-related factors are the procurement from deceased donor or from DCD (donation after cardiac death). DGF rate is about 30.8% in US deceased donors which is significantly higher in DCDs (45–55.1%) [11]. Other important parameters are the increase of age, the body mass index, the cause of death (anoxia vs. cerebrovascular), and diabetes. From a recipient point of view, the most important risk factors are related to body mass index, previous transplant, diabetes, and male gender.

Interventions in recipients, such as dopamine and superoxide dismutase infusions, have not been entirely successful in mitigation DGF. Donor management investigation has been less well studied but may be a critical opportunity to improve organ quality. Interestingly, optimal organ preservation prior to graft appears significant in order to reduce to possibility of DGF [12]. The use of advanced therapies, such as AMT could be useful in the amelioration of organ storage, protecting the tubular epithelial cells from mitochondria damage.

Static Cold Storage

The current golden standard for organ preservation is the static cold storage (SCS). SCS is a well-established and standardized procedure in three sterile plastic bags in

ice filled with preservation solution in bag 1 or saline/ringer lactate/other in bags 2/3 [9] (fig.10). SCS preservation allows for significant metabolic depression; cellular metabolism is reduced by 50% for every 10 °C decreases in temperature, and at standard SCS storage temperatures between 0 °C and 4 °C, cellular metabolism occurs at 10%-12% of baseline function [10]. The major advantage of SCS is the safety of the procedure. Basically nothing can happen to an organ in plastic bags, on ice, in a plastic container. However, as the metabolism in the organ is slowed but continues, the organ eventually can be damaged by the accumulation of succinate and reactive oxygen species (ROS). Is important to underlying that the immediate exposure to normothermia and oxygen could result in an inflammatory response and organ-specific damage early after transplantation [11], [12]. Moreover, in DCD and IRI after reperfusion is significantly pronounced and more detrimental than other types of organs [13]. Although advancements made in preservation solutions alone have resulted in superior patient outcomes and long-term graft function, the evolution of machine perfusion has culminated in protective capacities that go beyond those offered by SCS.



Michelotto, J., Gassner, J.M.G.V., Moosburner, S. *et al.* Ex vivo machine perfusion: current applications and future directions in liver transplantation. *Langenbecks Arch Surg* **406,** 39–54 (2021). https://doi.org/10.1007/s00423-020-02014-7

Figure 10. Comparison among static cold storage (SCS), Hypothermic machine perfusion (HMP), and normothermic machine perfusion (NMP).

Hypothermic Machine Perfusion

The idea behind the hypothermic machine Perfusion (HMP) is the preservation of the graft using cryoprotective mixtures in a metabolically depressed, but active, state (fig. 10). HMP continuously provides metabolic substrates for the generation of ATP, and thus enables the graft to restore tissue energy [14]. Although devices vary in structure from organ to organ, the essential device components are perfusate delivery, the equipment for monitoring the physiologic parameters, and a temperature regulation apparatus. The gold standard machine preservation solution is UW solution, which contains a collection of enzymes, energy substrates, and salts. Numerous studies have demonstrated a better early graft function and superior long-term patient outcomes in kidneys preserved with HMP over SCS. There are also evidences that although SCS is initially cheaper than HMP, when the expenses associated with ECD kidney are included in cost assessments, preservation with HMP may reduce overall healthcare costs and provide an economic benefit to transplant centres. Recently, another form of HMP gain attention, the hypothermic

oxygenated machine perfusion (HOPE). HOPE is a form of HMP that utilizes an oxygenated preservation solution, typically Belzer solution. Originally developed to protect graft cells against IRI, HOPE has been shown to improve post-transplant outcomes in DCD donors. However, the use of oxygen could be a problem, as slowing the rate of organ decline requires a supply of oxygen sufficient to meet the metabolic demands of the hypothermically preserved organ but below the level at which ROS production exceeds its rate of elimination [15].

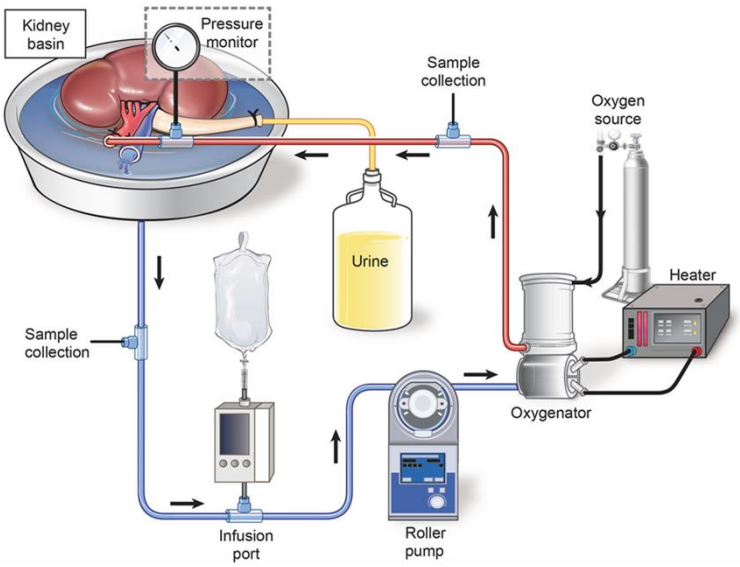
The most important disadvantages of HMP are associated with the complications and undesired side effects of continuous pump-dependent perfusion and the limitations for functional assessment under hypothermic conditions. Perfusion systems generate intravascular pressures that can predispose grafts to edema and microvascular damage with potentially serious consequences. Moreover, the high force of the perfusion system can generate excessively high glomerular pressures resulting in damage to the fenestrated glomerular epithelium and subsequent protein loss. Another significant risk is the arterial damage during HMP [16].

Normothermic Machine Perfusion

Normothermic machine perfusion (NMP) is functionally similar to HMP (fig. 10); the most important difference is the temperature of functioning, much more similar to a physiological condition. Comparably to HMP, NMP requires establishing circuits between the NMP device and the graft via separate arterial and venous shunts. Rotary or peristaltic pumps circulate an erythrocyte-based preservation solution through the circuits, while a membrane oxygenator performs oxygenation

and ventilation on the circulating fluid. One very important advantage compared to HMP is that NMP utilizes auto-regulatory mechanisms that allow for precise control of parameters like temperature, pressure, flow speed, and flow resistance. Furthermore, NMP provides a full functional assessment of a procured organ under physiologic conditions, typically between 34 °C and 39 °C. This feature makes NMP not only an invaluable tool for ex vivo functional evaluation but also a highly attractive means of expanding the eligible donor pool [17] (fig. 11).

Organ preservation with NMP offers several of the same advantages as HMP such as nutrient delivery and removal of nitrogenous waste products and CO_2. Only NMP, however, allows for graft assessment and therapeutic drug delivery under physiologic conditions, unique features that are especially critical for better preservation of the organs, especially marginal donor ones. There is also evidence that organ assessment under physiologic conditions provides for a more accurate functional evaluation and superior predictability of early graft function than HMP.



M.F. Blum, et al. Comparison of normothermic and hypothermic perfusion in porcine kidneys donated after cardiac death, Journal of Surgical Research. https://doi.org/10.1016/j.jss.2017.04.008.

Figure 11. General scheme of normothermic machine perfusion. The kidney is connected via separate arterial and venous shunts. A rotary or peristaltic pumps circulate an erythrocyte-based preservation solution through the circuits, while a membrane oxygenator performs oxygenation and ventilation on the circulating fluid.

Leicester's group was the first to translate NMP into clinical reality on a large scale. Eighteen ECD kidneys were subjected to NMP reconditioning prior to transplantation. Outcomes were compared to 47 matched ECD kidneys preserved by SCS. In this study, NMP resulted in a significantly reduced DGF rate compared to controls [18]. The work of Leicester et al., together with other clinical reports, demonstrated the potentiality of kidney HMP, especially in the preservation of marginal grafts, including grafts from DCD and ECD, with superior outcomes in the early phase compared to SCS.

As already mentioned, functional assessment under physiologic conditions is an important advantage of preservation with NMP; however, identifying biomarkers that accurately predict organ viability will be a crucial barrier to be overcome before the full potential of NMP can be realized.

References

[1] "Explore Human Anatomy, Physiology, and Genetics | Innerbody." https://www.innerbody.com/htm/body.html (accessed Oct. 21, 2021).

[2] O. Benjamin and S. L. Lappin, "End-Stage Renal Disease," *StatPearls*, Sep. 2021, Accessed: Oct. 19, 2021. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK499861/.

[3] J. Augustine, “Kidney transplant: New opportunities and challenges,” *Cleve. Clin. J. Med.*, vol. 85, no. 2, pp. 138–144, 2018, doi: 10.3949/ccjm.85gr.18001.

[4] R. Saran *et al.*, “US Renal Data System 2019 Annual Data Report: Epidemiology of Kidney Disease in the United States,” *American Journal of Kidney Diseases*, vol. 75, no. 1. pp. A6–A7, 2020, doi: 10.1053/j.ajkd.2019.09.003.

[5] A. C. Webster, E. V Nagler, R. L. Morton, and P. Masson, “Chronic Kidney Disease,” *The Lancet*, vol. 389, no. 10075. pp. 1238–1252, 2017, doi: 10.1016/S0140-6736(16)32064-5.

[6] C. W. Yang *et al.*, “Global case studies for chronic kidney disease/end-stage kidney disease care,” *Kidney International Supplements*, vol. 10, no. 1. Kidney Int Suppl (2011), pp. e24–e48, Mar. 01, 2020, doi: 10.1016/j.kisu.2019.11.010.

[7] R. A. Metzger, F. L. Delmonico, S. Feng, F. K. Port, J. J. Wynn, and R. M. Merion, “Expanded criteria donors for kidney transplantation,” in *American Journal of Transplantation*, 2003, vol. 3, no. SUPPL. 4, pp. 114–125, doi: 10.1034/j.1600-6143.3.s4.11.x.

[8] I. Reperfusion, “Ischemia/Reperfusion,” vol. 7, no. 1, pp. 113–170, 2017, doi: 10.1002/cphy.c160006.Ischemia/Reperfusion.

[9] G. M. Collins, M. Bravo-Shugarman, and P. I. Terasaki, “Kidney preservation for transportation. Initial perfusion and 30 hours’ ice storage.,” *Lancet*, vol. 2, no. 7632, pp. 1219–1222, 1969, doi: 10.1016/s0140-6736(69)90753-3.

[10] M. H. J. Maathuis, H. G. D. Leuvenink, and R. J. Ploeg, “Perspectives in organ preservation,” *Transplantation*, vol. 83, no. 10. Transplantation, pp. 1289–1298, May 2007, doi: 10.1097/01.tp.0000265586.66475.cc.

[11] H. SA, van H. E, and N. ML, “Normothermic machine perfusion of the kidney: better conditioning and repair?,” *Transpl. Int.*, vol. 28, no. 6, pp. 657–664, Jun. 2015, doi: 10.1111/TRI.12319.

[12] E. T. Chouchani *et al.*, “Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS,” *Nat. 2014 5157527*, vol. 515, no. 7527, pp. 431–435, Nov. 2014, doi: 10.1038/nature13909.

[13] C. D. Collard and S. Gelman, “Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury,” *Anesthesiology*, vol. 94, no. 6. Anesthesiology, pp. 1133–1138, 2001, doi: 10.1097/00000542-200106000-00030.

[14] D. Nakajima *et al.*, “Hypothermic machine perfusion ameliorates ischemia-Reperfusion injury in rat lungs from non-Heart-Beating donors,” *Transplantation*, vol. 92, no. 8, pp. 858–863, Oct. 2011, doi: 10.1097/TP.0b013e31822d8778.

[15] H. Groen *et al.*, “Cost-effectiveness of hypothermic machine preservation versus static cold storage in renal transplantation,” *Am. J. Transplant.*, vol. 12, no. 7, pp. 1824–1830, Jul. 2012, doi: 10.1111/j.1600-6143.2012.04030.x.

[16] M. L. Cobert, L. M. West, and M. E. Jessen, “Machine perfusion for cardiac allograft preservation,” *Current Opinion in Organ Transplantation*, vol. 13, no. 5. Curr Opin Organ Transplant, pp. 526–530, Oct. 2008, doi: 10.1097/MOT.0b013e32830fdf9a.

[17] A. S. Barbas and S. J. Knechtle, “Expanding the Donor Pool With Normothermic Ex Vivo Liver Perfusion: The Future Is Now,” *American Journal of Transplantation*, vol. 16, no. 11. Am J Transplant, pp. 3075–3076, Nov. 01, 2016, doi: 10.1111/ajt.13959.

[18] S. A. Hosgood, “Renal transplantation after ex vivo normothermic perfusion: The first clinical study,” *Am. J. Transplant.*, vol. 13, no. 5, pp. 1246–1252, May 2013, doi: 10.1111/ajt.12179.

5. *BOOK CHAPTER*: "MITOCHONDRIA TRANSPLANTATION IN ORGAN DAMAGE AND REPAIR"

Organ Repair and Regeneration: Preserving Organs in the Regenerative Medicine. Orlando et al. 2021

Andrea Rossi, Paolo Porporato, and Benedetta Bussolati

CHAPTER

13

Mitochondria transplantation in organ damage and repair

Andrea Rossi, Paolo Porporato, Benedetta Bussolati

Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy

Introduction

From the biochemical standpoint, mitochondria are known as the cellular powerhouse, being the main activity of this organelles is the ATP generation via oxidative phosphorylation. Nevertheless, this definition is somewhat limiting, shadowing the complexity of biological activities performed by mitochondria. Mitochondria, indeed, are involved in several activities, including proliferation, differentiation, and cell death [1]. Since 3 decades, they have been identified as the trigger of programmed cells death, driven by the release of cytochrome C from the mitochondrial membrane [2]. Recently, mitochondria activity, and in particular the rate of oxidative phosphorylation, has been correlated to cellular differentiation, while, in turn, its inhibition supports pluripotent stem cell generation [3]. Finally, several observations linked dysfunctional mitochondrial to aging and major systemic failure [4–6].

Mitochondria are surrounded by a double membrane system, necessary to the generation of an electrochemical proton gradient. This particular conformation allows the local accumulation of several elements, such as metabolites, proteins, reactive oxygen species, and Ca^{2+}, thus allowing signaling and biochemical reaction compartmentalization. Mitochondria are, therefore, influencing cellular biology by providing a wide range of metabolites and cofactor via the Krebs cycle, by regulating cellular Ca^{2+} levels, and reactive oxygen species (ROS) homeostasis linked to the plethora of redox reactions performed during ATP synthesis. Several metabolites generated by mitochondria present moonlighting activates, being more than metabolic intermediates, but acting as well as signaling molecules. For instance, the TCA cycle intermediate α-ketoglutarate is also a necessary substrate of hydroxylases, such as PHD2 or TET enzymes, thus altering gene expression [7]. The impact of some metabolites on tissue reprogramming is so important that the simple accumulation of such metabolites is sufficient to promote tumor transformation. Hence, TCA cycle intermediates, such as succinate and fumarate, are currently defined as oncometabolites [1]. Another critical aspect of mitochondrial biology is organelles dynamics. While often considered as immobile bean-shaped organelles, mitochondria are highly dynamic, undergoing the process

Organ Repair and Regeneration
https://doi.org/10.1016/B978-0-12-819451-5.00001-9

of fusion and fission, thus forming a diverse array of structures, from long filaments to donut-shaped structures. Intriguingly, it is clear that such networks control cellular function and tissue homeostasis. For instance, cellular differentiation has been linked to the ability of mitochondria to form fused networks as the deletion of the main mediator of such a cellular process (i.e., mitofusins) results in lethal phenotype linked to the impaired differentiation of embryonic cells into cardiomyocytes [8].

Intriguingly, a novel standpoint of mitochondrial dynamics is currently emerging, following the discovery that mitochondria can pass between cells, rescuing metabolic dysfunctions. Defining the biological processes driving this cellular cross-talk will have vast implications in several pathological settings, including fibrosis, septic shock, and organ damage [9]. In parallel, the exploitation of this biological property by supplying mitochondria to cells is currently on study in various approaches of regenerative medicine.

Role of mitochondria in organ damage

Considering the complex and central function of mitochondria in cell physiology described earlier [10], the better understanding of alteration in their metabolism during or even before organ damage results mandatory to better characterize the evolution of diseases and the possible therapeutic approaches.

Even if the metabolic patterns can strictly vary among the organs [11], several pieces of evidence suggest that the disruption of mitochondria architecture is one of the essential hallmarks of organ failure. More than the simple reduction of the ATP intake, the alteration of mitochondrial homeostasis can lead to the production of toxic species, as ROS or other reactive molecules, which can maximize the early tissue damage. Furthermore, the altered production of important factors, as heme and iron metabolites proper of mitochondria metabolism, can lead to an even bigger alteration in tissue function. Interestingly, mitochondria are particularly susceptible to variation of the extracellular and intracellular microenvironment: altered concentration in Ca^{2+} and Fe^{3+} has been reported to enhance mitochondrial damage [12,13]. Therefore, it appears evident that mitochondrial alterations are strictly correlated with most of the clinical conditions that lead to organ failure. In this section, we will focus on the role of mitochondria during damage, in particular ischemia/reperfusion injury, and on the subsequent effects of mitochondrial bioproducts, such as mitochondrial DNA, as immune-activators.

Ischemia/reperfusion injury

Ischemia/reperfusion injury (IRI) is a critical condition associated with a wide range of diseases, including myocardial damage, heart failure, cerebral and gastrointestinal dysfunction, and acute and chronic organ failure [13]. Moreover, IRI often occurs during kidney and liver transplantation [14]. IRI can be subdivided into two distinct phases. In the first, known as ischemia stage, the impairment of blood flow or the decrease of energetic supply leads to severe hypoxia condition. This dramatic alteration induces a switch to anaerobic metabolism and to the production of ROS, toxic either for mitochondria and for the whole cell [15]. As a consequence, the reduction of the ATP level and the change in pH alter the Ca^{2+} concentration, unsettling the ion balance inside the cell. Notably, the second stage, called reperfusion stage, can amplify the induced damage: the rapid restoration of reperfusion triggers several mechanisms, including immune system stimulation and apoptosis activation, which can surge the previous ROS and hypoxic damage to a clinical stage [16]. The detrimental effect of IRI is particularly evident in myocardial reperfusion injury occurring after coronary heart disease [16]. In kidney transplantation, IRI may occur as a consequence of brain death in the cadaveric donor or during blood flow reconstruction [17]. In the brain, one

of the most sensible organs to IRI, the damage is strictly correlated to the specificity of the brain's mitochondria [17]. In neurons, indeed, these organelles contain a lower amount of cytochrome C oxidase and a higher concentration of polyunsaturated fatty acids than in the other organs, making the brain more vulnerable to oxidative damage [17]. In general, however, the ischemia phase alters the electrochemical gradient established among the mitochondrial membranes, increasing ROS production and decreasing the activity of antioxidant enzymes [16]. The subsequent rapid reintroduction of O_2 during the reperfusion phase can greatly increase the ROS production, leading to the alteration of NADH/NAD and GSH/GSSG ratio [18]. These events together with the release of Ca^{2+} from the intramitochondrial space tend to activate the intrinsic pathway of apoptosis [18]. Taking together, it appears evident that mitochondria alteration during hypoxia can greatly amplify organ damage, especially during the reperfusion phase.

Effect of mitochondria as immune-system activators

Mitochondria are capable to form proteins using a proper translational system. Some of these proteins contain domains not recognized as "self" by the immune system, as N-formylated peptides, potent immune-activators [19]. Other than that, mitochondria released following tissue damage could act as damage-associated molecular patterns (DAMPs), initiating and perpetuating the inflammatory response [19,20]. As a proof of concept, Lin et al. recently demonstrated that the presence of circulating mitochondria might correlate with allograft rejection in organ donors [20]. In fact, in a murine model of allogeneic cardiac transplant, the injection of purified mitochondria increased the number of $CD4^+$ and $CD8^+$ lymphocytes, and the activation of endothelial and dendritic cells [20]. However, it is important to underline that injected isolated mitochondria were resuspended in PBS and injected the day after the purification. Considering this, reasonably the immune system might be activated due to the presence of damaged and disrupted mitochondria more than simply of isolated mitochondria.

Proinflammatory effect of mitochondrial DNA

Differently to all the other organelles present in the cell, mitochondria possess their own circular genome, so-called mitochondrial DNA (mtDNA), containing 37 genes encoding for mRNA, tRNA, and ribosomal RNA. mtDNA is a relatively small circular DNA containing some very peculiar structural differences with the nuclear DNA. Firstly, mtDNA follows different structural pathway: is still unclear if it contains some methylated GpC sequences (cytosine nucleotide followed by a guanine nucleotide) as the nuclear DNA or different methylated bases, [21–23]. A small part of the mtDNA is organized in a stable three-loop structure, in a similar way to the active transcriptional sequences present in the nuclear DNA [24]. Moreover, mtDNA lacks introns and exons and it is packaged in a very particular protein-DNA structure, similar to bacterial DNA and present only in the mitochondria [25]. mtDNA, present in the cytoplasm after cell autophagy or released by injured cells, was shown to be able to activate intracellular inflammation and innate immune response through Toll-like Receptor (TLR) 9. For instance, mtDNA increased the mRNA levels of NF-κB, IκB-α, and TLR9, activating the inflammasome response through the p38 MAP kinase pathway during lung injury [26].

On the other side, mtDNA release is part of the so-called mitochondrial alarmin, or DAMPs, a series of patterns able to activate and/or stimulate the innate immune system [24,27]. In such sense, Zang et al. demonstrated that inoculation of mtDNA can significantly increase the level of inflammation and the production of TLR 9 in the lungs of treated rats, compared with controls [26].

mtDNA as biomarker

Another important mechanism of immune signaling involves the presence of mtDNA in extracellular vesicles (EVs) [28,29]. EVs are a wide population of membranous microparticles released by all the different types of cells present in the body [30]. Other than signaling functions, EVs can activate the innate and adaptive immune system either in cancer and in case of cell stress [31]. Even if not well studied, the presence or increase of mtDNA in the EVs seems to contribute to this mechanism. Tong et al., in 2017, observed that in preeclampsia, a placental pathology with unclear pathogenesis, the amount of mtDNA in EVs is increased. Moreover, the presence of antiphospholipid antibodies, a particular class of antibodies that strongly increase the risk of preeclampsia [32], correlated with the presence of mtDNA in EVs and with endothelial cell activation, presumably through TLR 9 [32].

Increased concentration of mtDNA also correlated with intensive care unit (ICU) patients and with ICU mortality [33]. Nakahira et al. showed that the high presence of mtDNA (assessed by the measurement of NADH dehydrogenase 1 gene) correlated with the mortality rate in ICU patients and could be considered a predictive biomarker in hospitalized patients [33,34].

Mitochondrial transfer in organ repair

It is known that cells can destroy and then recycle damaged mitochondria, in a process called mitophagy [35]. In fact, considering that mitochondria do not possess an efficient DNA-system repair [36], mutations can accumulate and irreversibly damage firstly the mitochondria itself and then the other organelles. Moreover, damaged mitochondria can be replaced: a donor cell can transfer its own mitochondria to a damaged host cell, to replace and repair the mitochondria network [37]. In 2004, Rustom et al. firstly observed that injured cells, more than simply repair the damaged mitochondria, can acquire them from other donor cells [38]. In 2006, Spees et al. demonstrated that the transfer of mitochondria can rescue the aerobic respiration in mammalian cells lacking functional mitochondria [39]. Finally, in 2012, the work of Islam et al. not only validated in vivo the previous research but also identified stem cells, specifically bone marrow–derived stromal stem cells, as a physiologic source of transferred mitochondria in an in vivo model of acute lung injury [9].

Physiological mitochondrial transfer

Transfer of mitochondria is part of the physiological maintenance of the organ homeostasis, and it can occur through different mechanisms [40] (Fig. 13.1). Islam et al. reported that, in an bacterial lipopolysaccharide (LPS)-induced model of lung injury, the presence of mesenchymal stromal cells (MSC) from bone marrow was able to reduce the alveolar leukocytosis and protein leak, whereas mitochondria from dysfunctional MSC could not achieve this result. Moreover, the presence of MSC mitochondria was confirmed by optical evaluation and assessment of human mtDNA genes in the treated mouse lung [9]. In 2015, Phinney et al. brilliantly linked mitophagy in MSCs with the transfer of mitochondria to macrophages through a particular subgroup of vesicles. They were able to demonstrate that MSCs uploaded mitochondria via arrestin domain-containing protein 1-mediated MVs. The MSC vesicles were, then, engulfed by macrophages and utilized to produce energy. Furthermore, they observed that the exchange of microRNAs between macrophages and MSCs were able to downregulate the TLR expression and inflammatory signaling. This may represent one of the possible systems used by host cells to allow the entrance and the absorption of external mitochondria [41].

Another form of physiological mitochondria transfer is through tunneling nanotubes (TNTs) [38,42]. TNTs are membranous protrusions containing a skeleton able to tightly fuse the donor and the host cell. This type of transfer is involved in cell repair, activation of immune response, and

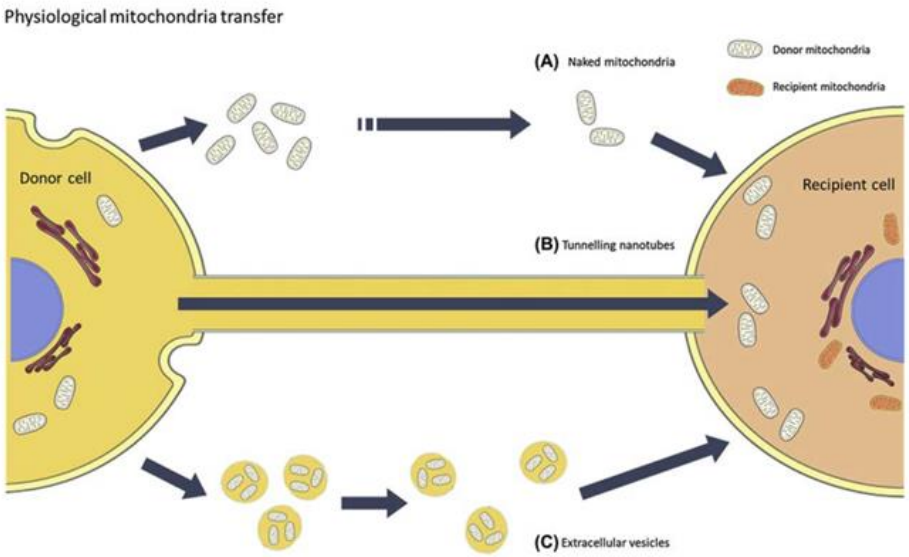


FIGURE 13.1 Physiological mitochondria transfer. The figure illustrates three different mechanisms of mitochondrial transfer: (A) directly released in the bloodstream or in the extracellular microenvironment in response to cellular stress, with activation of the immune system and of the inflammatory response; (B) tunneling nanotubes (TNTs), membranous protrusion involved in cell repair, activation of immune response, and cell reprogramming; (C) transfer inside extracellular vesicles (EVs). This transfer mechanism was observed mainly in MSCs, activating repair of cellular damage or enhancing phagocytic properties of cells.

cell reprogramming [42–44]. However, the directionality of the transfer in TNTs is still unclear.

Mitochondria can be, also, released naked, in response to cellular stress [40]. Various studies reported that both apoptotic hepatocytes and activated platelets can expel viable mitochondria, perhaps activating the immune system and the inflammatory response [45,46]. However, the mechanisms underlying the preference between one or another transfer is still unclear.

Artificial mitochondrial transfer for organ repair

As mitochondria transfer is a physiological mechanism used to repair or reprogram damaged cells, various groups tried to mimic this process artificially, to elaborate a new possible therapeutic approach (Fig. 13.2). To date, several groups are working on what is called artificial mitochondria transfer (AMT). The first demonstration of an AMT was obtained by Clark et al. in 1982, by simple coincubation. They observed that antibiotic-resistant genes carried by mtDNA present in isolated mitochondria suspended in the medium passed to sensitive host cells [47]. Moreover, the microinjection of mitochondria in the human cell was able to replace damaged mitochondria [48]. A possible application was shown by the possibility to replace mitochondria in fertilized mice eggs [49]. More recently, the artificial coincubation of extracted fully respiration-competent and fresh mitochondria emerged as a valid possible option in the field of regenerative medicine [50–53].

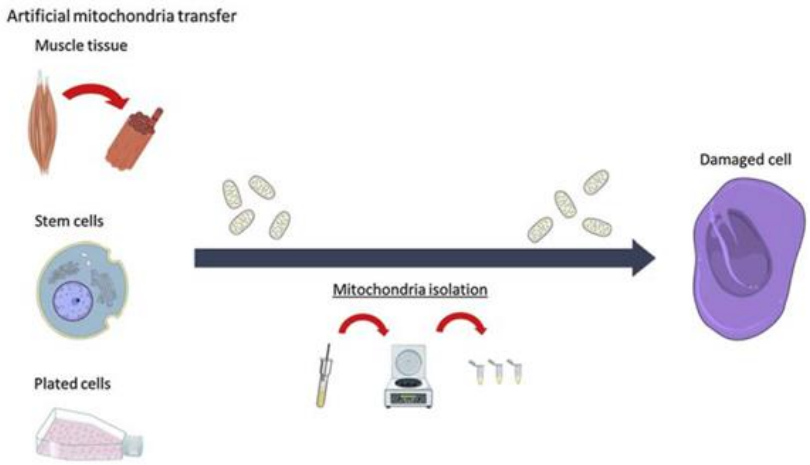


FIGURE 13.2 General scheme of artificial mitochondria transfer. Mitochondria can be isolated from differentiated organs and tissues, stem cells, as mesenchymal stem cells or differentiated cells. The isolation usually consists of three steps: homogenization of the sample via mechanical strokes; centrifugations, to remove broken parts, and isolation of the mitochondrial pellet, resuspended in a specific buffer. The isolated mitochondria are then injected and internalized by damaged host cells.

In recent years, McCully and his group worked on AMT as a therapeutic approach in ischemic cardiomyocytes, both in vitro and in vivo models [53,54]. Indeed, injection of isolated mitochondria was able to protect ischemic cardiac cells during the reperfusion, in an in vivo model of rabbit myocardial IRI injury [53]. The molecular mechanism that allows the internalization of these organelles in the recipient cells was ascribed to an ATP-dependent mechanism leading to mitochondria endocytosis [55]. Other studies showed, using three-dimensional superresolution microscopy and transmission electron microscopy, that mitochondria are incorporated in cardiac cells within minutes. Inside the cell, the acquired organelles are transported to endosomes or lysosomes and, then, fuse with the mitochondria network [56]. Recently, an AMT using autologous muscle-derived mitochondria was performed in a clinical setting to treat pediatric patients affected by myocardial ischemia-reperfusion injury. All patients did not show any adverse short-term effects and demonstrated improvement in ventricular function [54].

Another possible setting for the application of AMT is schizophrenia (SZ), as isolated active mitochondria were able to differentiate SZ-derived induced pluripotent stem cells into glutamatergic neurons. Moreover, in a rodent model of SZ, the injection of mitochondria into the intra-prefrontal cortex of adolescent mice prevented attentional deficit at adulthood [51]. This last work showed that AMT could be also used to commit undifferentiated cells, an extremely attractive aspect in the treatment of organ as the brain is.

The potentiality of mitochondrial transfer as a therapeutic option has been tested also in the liver [57], and in an in vivo model of diabetic nephropathy [58]. Our group is also testing the potentiality of mitochondria transfer in a model of acute kidney injury induced by cisplatin. Cisplatin, a normally used chemotherapy drug,

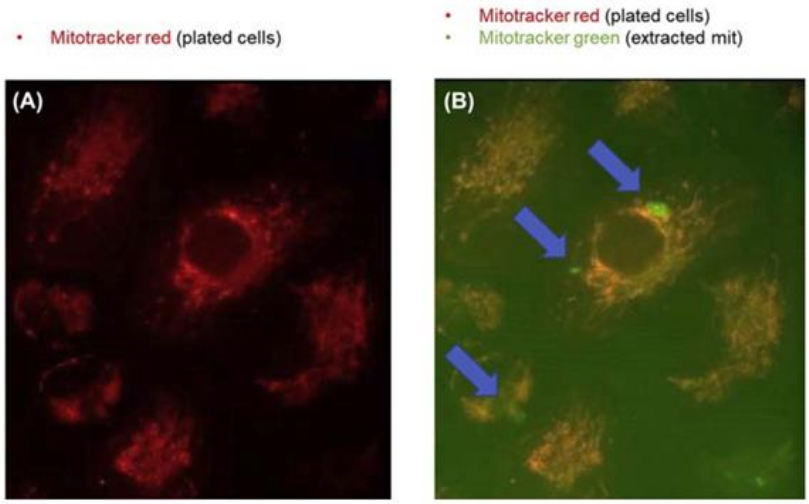


FIGURE 13.3 Optical observation of mitochondrial transfer. Proximal tubular renal cells were plated and dyed with mitotracker red. After the induction of damage, previously isolated mitochondria, obtained from the same cell type, were coincubated for 24 h. *Blue arrows* show the isolated mitochondria (*stained in green*) internalized by plated cells.

tend to accumulate in active mitochondria, causing toxicity. We observed that human proximal tubular cells damaged with cisplatin can internalize isolated fully viable mitochondria (Fig. 13.3).

Technical considerations

A critical point for every possible mitochondrial transplantation therapy regards the isolation of fully viable and respiration-competent mitochondria (Fig. 13.2). As isolated mitochondria are particularly sensible to different factors that may alter the membrane potential or disrupt the external membrane, the transfer requires "fresh" isolated mitochondria. This, therefore, implies that extraction should be completed immediately before use [59] and that freezing is not allowed as it would disrupt the outer membrane. Moreover, the extraction is conventionally performed in a sucrose/mannitol enriched medium, isosmotic and with physiologic pH. Temperature variation is another parameter to take into consideration and isolated mitochondria are generally kept in ice at 4°C to preserve their structural characteristics [52,58,60]. Considered all these parameters, it appears evident why isolation protocols are focused on timing and preservation of viability during the isolation, more than on the purity of extraction.

Another important parameter is the number of mitochondria used for AMT. There is great heterogeneity in the literature among the quantity of mitochondria. It is important to consider that the cell source could influence the number of mitochondria used per host cells [61]. Another aspect is represented by the recipient cells and by the in vitro/in vivo type of experiment. In the in vivo case, for instance, 2×10^5 mitochondria per gram tissue wet weight have been described [59]. However, to quantify the amount of mitochondria through their number could be misleading, as mitochondria continuously tend

to fuse and fix together. Moreover, a great number of labeled mitochondria could be stacked together during the counting. For these reasons, various groups tend to quantify the protein amount of the extracted mitochondria network, more than the number [62]. Furthermore, this process is easier, quicker, and allows a faster quantification during the extraction.

Conclusion

It appears evident that mitochondria represent a pivotal organelle in the context of organ damage and repair. However, modulation of mitochondrial activity or targeting of mitochondrial proteins is still to be improved. Mitochondria transplantation is emerging as a plausible therapeutic approach in various fields of applied medicine. In such sense, AMT, through the substitution, rather than targeting of the damaged mitochondria, emerged as one of the most promising approaches. One of the other strengths of AMT is the versatility: AMT was successfully used in several neurological diseases, as Parkinson's disease [63] or traumatic brain or spinal cord injury [60,64]; as well as in organ ischemia [59], as mentioned before. However, various topics still require investigation Firstly, the mechanisms describing how mitochondria can freely enter the damaged cell, and how can they survive to the high Ca^{2+} concentration present in the bloodstream are unknown. The second main issue is related to the source of mitochondria to be used for transplant. Most of the studies highlighted that, in physiological conditions, all the mitochondria donor cell types are stem cells. MSCs have therefore up to date, considered the preferential source for isolated mitochondria, also in virtue of their role in tissue repair [58]. However, stem cells exhibit low mitochondria content and structural differences in comparison with more specialized cells. In such sense, various works demonstrated a beneficial effect of mitochondria derived from highly specialized cells, as muscle cells [59]. Lastly, secreted factors and exosome coisolated with mitochondria could contribute to the observed effects of AMT. Of relevance, the transplant of mitochondria has been already proven to be beneficial in a human application for cardiac IRI and other evaluations are urgently needed for safe and efficacious use of AMT in clinics.

References

[1] Porporato PE, Filigheddu N, Pedro JMBS, Kroemer G, Galluzzi L. Mitochondrial metabolism and cancer. Cell Res March 1, 2018;28(3):265–80. Nature Publishing Group.

[2] Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med May 2000;6(5):513–9.

[3] Folmes CDL, et al. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nu. Cell Metabol 2011;14(2):264–71.

[4] López-Otín C, Galluzzi L, Freije JMP, Madeo F, Kroemer G. Metabolic control of longevity. Cell August 2016;166(4):802–21.

[5] Fukawa T, et al. Excessive fatty acid oxidation induces muscle atrophy in cancer cachexia. Nat Med 2016;22(6):666–71.

[6] Wyart E, et al. Metabolic alterations in a slow-paced model of pancreatic cancer-induced wasting. Oxid. Med. Cell. Longev 2018;2018.

[7] Frezza C. Mitochondrial metabolites: undercover signalling molecules. Interface Focus April 2017;7(2):20160100.

[8] Kasahara A, Cipolat S, Chen Y, Dorn GW, Scorrano L. Mitochondrial fusion directs cardiomyocyte differentiation via calcineurin and Notch signaling. Science November 2013;342(6159):734–7.

[9] Islam MN, et al. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nat Med 2012;18(5):759–65.

[10] Krasnikov BF, Cooper AJL. Models, methods, and approaches to study mitochondrial functioning in vitro, in situ, and in vivo: editorial for the special issue on Mitochondrial Biochemistry and Bioenergetics. Anal Biochem 2018;552:1–3. no. April.

[11] Jassem W, Heaton ND. The role of mitochondria in ischemia/reperfusion injury in organ transplantation. In: Kidney international; 2004.

[12] Crompton M. The mitochondrial permeabitity transition pore and its role in cell death. Biochem J 1999;341(2):233–49.

[13] Wu MY, et al. Current mechanistic concepts in ischemia and reperfusion injury. Cell Physiol Biochem 2018;46(4):1650−67.
[14] Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: pathogenesis and treatment. World J Transplant 2015;5(2):52.
[15] Maneechote C, Palee S, Chattipakorn SC, Chattipakorn N. Roles of mitochondrial dynamics modulators in cardiac ischaemia/reperfusion injury. J Cell Mol Med 2017;21(11):2643−53.
[16] Honda HM, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. Ann N Y Acad Sci 2005; 1047:248−58.
[17] Adibhatla RM, Hatcher JF. Lipid oxidation and peroxidation in CNS Health and disease: from molecular mechanisms to therapeutic opportunities. Antioxidants Redox Signal 2010;12(1):125−69.
[18] Costantini P, Chernyak BV, Petronilli V, Bernardi P. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 1996;271(12): 6746−51.
[19] Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. Trends Immunol 2002;23(11):541−8.
[20] Lin L, et al. Circulating mitochondria in organ donors promote allograft rejection. Am J Transplant 2019; 19(7):1917−29.
[21] Bellizzi D, et al. The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern. DNA Res 2013;20(6):537−47.
[22] Pollack Y, Kasir J, Shemer R, Metzger S, Szyf M. Methylation pattern of mouse mitochondrial DNA. Nucleic Acids Res 1984;12(12):4811−24.
[23] Hong EE, Okitsu CY, Smith AD, Hsieh C-L. Regionally specific and genome-wide analyses conclusively demonstrate the absence of CpG methylation in human mitochondrial DNA. Mol Cell Biol 2013;33(14): 2683−90.
[24] West AP, Shadel GS. Mitochondrial DNA in innate immune responses and inflammatory pathology. Nat Rev Immunol 2017;17(6):363−75.
[25] Grazioli S, Pugin J. Mitochondrial damage-associated molecular patterns: from inflammatory signaling to human diseases. Front Immunol 2018;9:1−17. no. MAY.
[26] Zhang JZ, Liu Z, Liu J, Ren JX, Sun TS. Mitochondrial DNA induces inflammation and increases TLR9/NF-κB expression in lung tissue. Int J Mol Med 2014; 33(4):817−24.
[27] Mehta MM, Weinberg SE, Chandel NS. Mitochondrial control of immunity: beyond ATP. Nat Rev Immunol 2017;17(10):608−20.
[28] Sansone P, et al. Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc Natl Acad Sci USA 2017;114(43): E9066−75.
[29] Puhm F, et al. Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce type I IFN and TNF responses in endothelial cells. Circ Res 2019;125(1):43−52.
[30] Pomatto MAC, Gai C, Bussolati B, Camussi G. Extracellular vesicles in renal pathophysiology. Front. Mol. Biosci 2017;4:1−22. no. JUN.
[31] Barros FM, Carneiro F, Machado JC, Melo SA. Exosomes and immune response in cancer: friends or foes? Front Immunol 2018;9. no. APR.
[32] Tong M, et al. Antiphospholipid antibodies increase the levels of mitochondrial DNA in placental extracellular vesicles: alarmin-g for preeclampsia. Sci Rep 2017;7(1):1−12.
[33] Nakahira K, et al. Circulating mitochondrial DNA in patients in the ICU as a marker of mortality: derivation and validation. PLoS Med 2013;10(12):1−12.
[34] Falah M, et al. The potential role for use of mitochondrial DNA copy number as predictive biomarker in presbycusis. Therapeut Clin Risk Manag 2016;12: 1573−8.
[35] Ding WX, Yin XM. Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem July 2012;393(7):547−64.
[36] Zinovkina LA. Mechanisms of mitochondrial DNA repair in mammals. Biochem 2018;83(3):233−49.
[37] Li C, et al. Mesenchymal stem cells and their mitochondrial transfer: a double-edged sword. Biosci Rep May 3, 2019;39(5). Portland Press Ltd.
[38] Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH. Nanotubular highways for intercellular organelle transport. Science 2004;303(5660):1007−10.
[39] Spees JL, Olson SD, Whitney MJ, Prockop DJ. Mitochondrial transfer between cells can rescue aerobic respiration. Proc Natl Acad Sci USA 2006;103(5): 1283−8.
[40] Caicedo A, Aponte PM, Cabrera F, Hidalgo C, Khoury M. Artificial mitochondria transfer: current challenges, advances, and future applications. Stem Cell Int 2017;2017.
[41] Phinney DG, et al. Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs. Nat Commun 2015;6:1−15.
[42] Sinha P, Islam MN, Bhattacharya S, Bhattacharya J. Intercellular mitochondrial transfer: Bioenergetic crosstalk between cells. Curr Opin Genet Dev 2016;38: 97−101. no. June.
[43] Jackson MV, et al. Mitochondrial Transfer via Tunneling Nanotubes is an Important Mechanism by Which Mesenchymal Stem Cells Enhance Macrophage

Phagocytosis in the In Vitro and In Vivo Models of ARDS. Stem Cells 2016;34(8):2210–23.
[44] Bukoreshtliev NV, Wang X, Hodneland E, Gurke S, Barroso JFV, Gerdes HH. Selective block of tunneling nanotube (TNT) formation inhibits intercellular organelle transfer between PC12 cells. FEBS Lett 2009;583(9): 1481–8.
[45] Dong JF. Platelet microparticles are not created equal. Blood 2014;124(14):2161–2.
[46] Nakajima A, Kurihara H, Yagita H, Okumura K, Nakano H. Mitochondrial extrusion through the cytoplasmic vacuoles during cell death. J Biol Chem 2008; 283(35):24128–35.
[47] Clark MA, Shay JW. Mitochondrial transformation of mammalian cells. Nature 1982;295(5850):605–7.
[48] King MP, Attardi G. Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. Cell Mar. 1988;52(6):811–9.
[49] Pinkert CA, Irwin MH, Johnson LW, Moffatt RJ. Mitochondria transfer into mouse ova by microinjection. Transgenic Res 1997;6(6):379–83.
[50] Berridge MV, Schneider RT, McConnell MJ. Mitochondrial transfer from astrocytes to neurons following ischemic insult: guilt by association? Cell Metabol 2016;24(3):376–8.
[51] Robicsek O, et al. Isolated mitochondria transfer improves neuronal differentiation of schizophrenia-derived induced pluripotent stem cells and rescues deficits in a rat model of the disorder. Schizophr Bull 2018;44(2):432–42.
[52] McCully JD, Cowan DB, Pacak CA, Toumpoulis IK, Dayalan H, Levitsky S. Injection of isolated mitochondria during early reperfusion for cardioprotection. Am J Physiol Heart Circ Physiol 2009;296(1):94–105.
[53] Masuzawa A, et al. Transplantation of autologously derived mitochondria protects the heart from ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2013;304(7):966–82.
[54] Emani SM, Piekarski BL, Harrild D, del Nido PJ, McCully JD. Autologous mitochondrial transplantation for dysfunction after ischemia-reperfusion injury. J Thorac Cardiovasc Surg July 2017;154(1):286–9.
[55] Pacak CA, et al. Actin-dependent mitochondrial internalization in cardiomyocytes: evidence for rescue of mitochondrial function. Biol Open 2015;4(5):622–6.
[56] Cowan DB, Yao R, Thedsanamoorthy JK, Zurakowski D, Del Nido PJ, Mccully JD. Transit and integration of extracellular mitochondria in human heart cells OPEN. Sci Rep 2017.
[57] Lin HC, Liu SY, Lai HS, Lai IR. Isolated mitochondria infusion mitigates ischemia-reperfusion injury of the liver in rats. Shock 2013;39(3):304–10.
[58] Konari N, Nagaishi K, Kikuchi S, Fujimiya M. Mitochondria transfer from mesenchymal stem cells structurally and functionally repairs renal proximal tubular epithelial cells in diabetic nephropathy in vivo. Sci Rep 2019;9(1):1–14.
[59] McCully JD, Levitsky S, del Nido PJ, Cowan DB. Mitochondrial transplantation for therapeutic use. Clin Transl Med 2016;5(1).
[60] Chien L, Liang M-Z, Chang C-Y, Wang C, Chen L. Mitochondrial therapy promotes regeneration of injured hippocampal neurons. Biochim Biophys Acta Mol Basis Dis 2018;1864(9 Pt B):3001–12.
[61] Azimzadeh P, Aghdaei HA, Tarban P, Akhondi MM, Shirazi A, Khorshid HRK. Comparison of three methods for mitochondria isolation from the human liver cell line (HepG2). Gastroenterol Hepatol Bed to Bench 2016;9(2):105–13.
[62] Kitani T, Kami D, Matoba S, Gojo S. Internalization of isolated functional mitochondria: involvement of macropinocytosis. J Cell Mol Med 2014;18(8): 1694–703.
[63] Chang JC, et al. Peptide-mediated delivery of donor mitochondria improves mitochondrial function and cell viability in human cybrid cells with the MELAS A3243G mutation. Sci Rep Dec. 2017;7(1).
[64] Gollihue JL, Rabchevsky AG. Prospects for therapeutic mitochondrial transplantation. Mitochondrion 2017;35: 70–9. no. March.

6. *PhD PROJECT*: "INJECTION OF ISOLATED RESPIRATION-COMPETENT MITOCHONDRIA AS THERAPEUTIC MODEL IN THE TREATMENT OF ISCHEMIA/REPERFUSION INJURY IN KIDNEY TRANSPLANTATION"

Introduction

Mitochondria transfer is a fascinating phenomenon triggered by stress signals, through which eukaryotic cells acquire new mitochondria from healthy donor cells, to ensure repair and survival after damage [1]. During this process, the mitochondria's small size and capacity to change their shape and length, allow them to be transported from donor to recipient cells by transporting mechanisms like tunneling nanotubes and microvesicles [2]. The organelles will eventually be incorporated into the endogenous mitochondrial network of the recipient cell that needs to be rescued, to restore its bio-energetic profile and health. As mitochondria transfer has been found to play a critical role in healing several diseases among which brain and cardiac injury, respiratory and renal disorders; artificial mitochondria transfer (AMT) has recently emerged as a promising therapeutic approach for conditions characterized by mitochondrial damage, including ischemia-reperfusion injury (IRI) complicating organ transplantation [1], [3]–[5]. In the IRI syndrome, hypoxia triggers a cascade of events that will eventually hit the mitochondria and may lead to cellular necrosis if not adequately treated. The

clinical consequences of these biomolecular events are devastating and may result in unsuccessful transplantation and graft loss. We, therefore, speculated that AMT may mitigate IRI through the replacement of damaged mitochondria with viable organelles deriving from healthy donor cells. We herein propose an *in vitro* and an *ex vivo* model of AMT in the setting of renal transplantation (RT). RT was chosen for two reasons. First, because it is the most frequently performed transplant worldwide; validation of our hypothesis with RT will warrant translation to all other solid organ transplants. Second, because of the dramatically increasing number of patients who require RT despite a still insufficient organ supply. According to our evidence, *in* vitro, AMT is able to protect human proximal tubular epithelial cells from ischemic damage, reducing cytotoxicity and restoring the cell proliferation capacity. Moreover, we observed a significant increase in the activity of important enzymes involved in tricarboxylic acid (TCA) cycle and oxidative phosphorylation, suggesting the improvement of mitochondrial function. *Ex vivo*, in a porcine model of kidney transplant preservation, AMT significantly reduced apoptosis, and decreased the presence of markers of kidney damage in the perfusate. Considering these findings, we proved that AMT could be considered as a potential therapeutic approach for solid organ transplantation.

7. MATERIAL AND METHODS

Cell Culture

HK-2 cells (ATCC) were cultured in DMEM low glucose (Euroclone) plus 10% FBS (Euroclone) at 37 °C in 5% CO_2. Human conditional immortalized proximal tubular cells (ciPTECs) were purchased from Cell4Pharma and cultured in DMEM F-12 (Gibco), supplemented with ITS (Sigma), hydrocortisone (Sigma), EGF (Sigma), tri-iodothyronine (Sigma) and 10% FBS (Euroclone). ciPTECs were immortalised using the temperature sensitive SV40ts A58 and human telomerase genes (hTERT), thus at 33 °C act as immortalized cells, but at 37 °C hTERT is inactivated and in 7 days they turn to primary cell. For the expansion phase, ciPTECs were cultured in 5% CO_2 at 33 °C. One week before experiments, ciPTECs were moved to 37 °C incubator and the medium was changed, in order to activate the switch from immortalized cells to primary cells.

In vitro Mitochondrial isolation protocol

The mitochondria isolation protocol is adapted from Konari et al. [6]. Briefly, plated cells were detached and pelleted by centrifugation at 2300 × g for 5 min. The cell pellet was resuspended in homogenization buffer containing 20 mM HEPES-KOH (pH 7.4), 220 mM mannitol, 70 mM sucrose and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at a density of 1.0×10^7 cells/mL and incubated on ice for 5 min. Next, cells were crushed by 60 strokes using a 27-gauge needle on

ice. Crushed cells were centrifuged three times at 400 × g for 5 min to remove unbroken cells. Isolated mitochondria were pelleted by centrifugation at 5800 × g for 5 min and resuspended in resuspension buffer (RB) (250 mmol/L sucrose, 20 mmol/L K^+-HEPES buffer [pH7.2], 0.5 mmol/L K^+-EGTA [pH 7.4]).

In vitro Experimental protocol

To induct IRI, primary ciPTECs were damaged using a mix of Antymic A (Sigma) and 2-deoxyglucose (Sigma) in serum-free complete medium for 4h. At the same time, mitochondria were isolated from healthy primary ciPTECs. At the end of the 4 hours, primary ciPTECs were washed twice in HBSS and co-incubated with isolated mitochondria resuspended in resuspension buffer (RB) or with RB alone in complete medium without FBS for 24h. ciPTECs were then washed three times and cultured with complete medium plus 10% FBS. After 72h the differences between treated and controls were evaluated.

Isolated mitochondria evaluations

To measure the ATP production on isolated mitochondria resuspended in RB, the luciferin-oxyluciferin reaction was evaluated using the CellTiter-glo 2.0 kit (Promega). This reaction utilises ATP emitting light. Thus, the light emission is directly proportional to ATP concentration. To evaluate their activity, isolated mitochondria were stained using MitoTracker red CMXRos (Invitrogen). Isolated mitochondria were co-incubated with MitoTracker red for 30 min, pelleted by

centrifugation, and resuspended in fresh RB twice to remove dye excess. Mitochondria internalization was evaluated labelling mitochondria with GFP. HK-2 mitochondria were infected with baculovirus encoding GFP-labelled E1alpha pyruvate dehydrogenase (BacMam2 delivery system, Life Technologies), a protein ubiquitously expressed in mitochondria. After isolation and co-incubation, the fluorescence was assessed optically.

Measurement of Proliferative Capability

The proliferative capability after AMT was assessed by measuring the total ATP concentration on primary ciPTECs plated in 96 well-plate and subjected to IRI alone (negative control) or to IRI and AMT (treated), using the CellTiter-Glo 2.0 kit (Promega). The BRDU proliferation assay (Sigma-Aldrich) was performed using the same experimental setting. For both ATP and BRDU assays the outcome was three days after mitochondrial treatment.

Evaluation of intracellular cytotoxicity

Intracellular cytotoxicity was evaluated using the Apotox-Glo Triplex Assay kit (Promega). Briefly, primary ciPTEC cells were subjected to IRI damage and then treated with mitochondria or with RB alone for 24h. After three days, the accumulation and cleavage of GF-AFC were assessed according to the manufacturer protocol.

Metabolic analysis

The analysis of ROS production, mitochondria membrane polarization, enzymes involved in TCA cycle and oxidative phosphorylation, ETC and ATP concentration were performed by prof. Riganti's lab from primary ciPTEC cells subjected to IRI and AMT according to the *in vitro* experimental setting. After 72h, cells were pelleted and immediately snap freeze. In the sections below the used methodologies are extensively explained.

Mitochondrial extraction

Cells were lysed in 0.5 ml mitochondria lysis buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM Mg Cl2, 1.8 mM ATP, 1 mM EDTA, pH7.2), supplemented with Protease Inhibitor Cocktail III (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM NaF. Samples were clarified by centrifugation at 650 g for 3 min at 4oC. Supernatants were collected and centrifuged at 13000 g for 5 min at 4oC. The new supernatants, corresponding to the cytosolic fraction, were used for cytosolic ROS measurements. Pellets, containing mitochondria, were washed once with lysis buffer and resuspended in 0.25 ml mitochondria resuspension buffer (250 mM sucrose, 15 mM K2HPO4, 2 mM MgCl2, 0.5 mM EDTA). 50 µl aliquots were sonicated and used for the measurement of protein content by the BCA Protein Assay kit (Sigma Alsrich, Milan, Italy) and for quality control: 10 µg of each sonicated sample were analyzed by SDS-PAGE and immunoblotting with an anti-porin antibody (Abcam, Cambridge, UK; clone 20B12AF2) to confirm the presence of mitochondrial proteins in the extracts. The remaining 200 µl were used for the metabolic assays reported below.

Mitochondrial damage

Mitochondrial reactive oxygen species (mt ROS). The mitochondria extracted from 1×10^6 cells were used for the assay. One 50 µl aliquot was sonicated and used to measure mitochondrial proteins. The remaining sample was treated for 30 min at 37 °C with 5 µM of the ROS-sensitive fluorescent probe MitoSOX (ThermoFisher, Waltham, MA). Samples fluorescence was measured using a HT Synergy 96-well micro-plate reader (Bio-Tek Instruments, Winooski, VT). The RFUs were converted into nanomoles ROS/mg mitochondrial proteins, according to a titration curve performed with serial dilutions of H2O2.

Mitochondrial tiobarbituric reactive substances (TBARS). The mitochondria extracted from 1×10^6 cells were used for the assay. One 50 µl aliquot was sonicated and used to measure mitochondrial proteins. The extent of oxidative damage in the remaining mitochondrial samples was measured in mitochondrial extracts by using the Lipid Peroxidation (4-HNE) Assay Kit (Abcam) that evaluates the 4-hydroxy-nonenale, one of the thiobarbituric reactive substances (TBARS) that is an index of lipid peroxidation. Results were expressed as nmol/mg mitochondrial protein.

Mitochondria depolarization. 1×10^6 cells were washed with PBS, detached by gentle scraping and incubated for 30 minutes at 37 °C with 2 µM of the fluorescent probe JC-1 (Biotium Inc., Hayward, CA), then centrifuged at 13000 × g for 5 minutes and re-suspended in 0.5 ml PBS. The red fluorescence (λ excitation: 550 nm, λ emission: 600 nm), index of polarized mitochondria, and the green fluorescence (λ excitation: 485 nm; λ emission: 535 nm), index of depolarized mitochondria fluorescence, were read using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments). The relative fluorescence units (RFUs) were used to calculate the percentage of green versus red mitochondria, considered an index of damaged mitochondria.

TCA cycle enzymes

The activities of citrate synthase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, were measured spectrophotometrically using the Citrate

Synthase Assay Kit (Sigma Aldrich), the Alpha Ketoglutarate (alpha KG) Assay Kit (Abcam), the Succinate Dehydrogenase Activity Colorimetric Assay Kit (BioVision, Milpitas, CA), the Malate Dehydrogenase Assay Kit (Sigma Aldrich), as per manufacturer's instructions. Results were expressed as U/mg proteins (citrate synthase) nmoles NADH/min/mg proteins (α-ketoglutarate dehydrogenase, malate dehydrogenase), nmoles FADH2/min/mg proteins (succinate dehydrogenase).

Oxidative phosphorylation (OXPHOS)

Electron transport chain complex (ETC). To measure electron flux from complex I to complex III, taken as an index of the mitochondrial respiratory activity, 50 µg of non-sonicated mitochondrial samples, isolated as indicated above, were re-suspended in 0.2 ml buffer A (5 mM KH2PO4, 5 mM MgCl2, 5% w/v bovine serum albumin, BSA; pH 7.2) to which 0.1 ml buffer B (25% w/v saponin, 50 mM KH2PO4, 5 mM MgCl2, 5% w/v BSA, 0.12 mM oxidized cytochrome c, 0.2 mM NaN3, which blocks complex IV allowing the accumulation of reduced cytochrome c; pH 7.5) was added for 5 min at room temperature. The cytochrome c reduction reaction was started by adding 0.15 mM NADH and was followed for 5 min at 37°C, reading the absorbance at 550 nm by a Packard microplate reader EL340 (Bio-Tek Instruments). The results were expressed as nanomoles of reduced cytochrome c /min/mg mitochondrial proteins.

ATP amounts in mitochondrial extracts were measured with the ATP Bioluminescent Assay Kit (Millipore Sigma), as per manufacturer's instructions. Results were expressed as nmoles/mg mitochondrial proteins.

Glutaminolysis. Cells were washed with PBS, detached by gentle scraping, centrifuged at 13,000 x g for 5 min at 4°C, re-suspended in 250 µl of buffer A (150 mM KH2PO4, 63 mM Tris/HCl, 0.25 mM EDTA; pH 8.6) and sonicated. A volume of 100 µl of the whole cell lysates was incubated for 30 min at 37°C in a quartz cuvette, in the presence of 50 µl of 20 mM L-glutamine and 850 µl of buffer B (80 mM Tris/HCl, 20 mM NAD+, 20 mM ADP, 3% v/v H2O2; pH 9.4). The absorbance of NADH was monitored at 340 nm using a

Lambda 3 spectrophotometer (PerkinElmer). The kinetics was linear throughout the assay. The results were expressed as µmol NADH/min/mg cell proteins and were considered as an index of the activity of glutaminase (GLS) plus L-glutamic dehydrogenase (GLUDH). In a second series of samples, 20 µL of the GLS inhibitor bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide BTPES (30 µM) was added after 15 min. This concentration was chosen as it produced 100% inhibition of GLS activity in our system (not shown). The absorbance of NADH was monitored for 15 min as described previously. The results, considered as an index of the activity of GLUDH, were expressed as µmol NADH/min/mg cell proteins. GLS activity was obtained by subtracting the rate of the second assay from the rate of the first one.

Ex vivo mitochondria isolation protocol

For the *ex vivo* experiments, mitochondria were isolated from gastrocnemius muscle immediately after kidneys procurement from the same animal. The amount of mitochondria used were $1x10^8$, according to literature [7], [8] and to our previous experiments. The isolation protocol was adapted from the work of Preble et al [9]. Briefly, 2 gr of muscle were isolated through tissue dissection and the cell debris were pelleted through a first centrifugation. The supernatant was then centrifugated at the mitochondria pellet suspended in 0.5 ml RB and used immediately for AMT.

Ex vivo experimental protocol

The *ex* vivo part of this work was developed at Wake Forest University (NC, USA) under the supervision of prof. Giuseppe Orlando's lab. Three male Yorkshire pigs, weighing on average 25 Kg, were euthanized for a total of six kidneys. In each

experiment, the paired kidneys were retrieved from the donor pig: one was subjected to the AMT, the other will be utilized as control. Immediately after the procurement, the renal artery and vein were cannulated, and a Luer-Lok connector of the appropriate size was inserted and affixed and rinsed with 500 ml of perfusate (UW solution). Then, kidneys were rewarmed to 37 C° in a warm water bath for 30 minutes to mimic the warm ischemia. After that, the renal vein was clamped, and a 10 ml of mitochondrial solution was injected into the renal artery for 30 min. One kidney was subjected to the AMT whereas the other was infused with RB alone as control. Lastly, kidneys were housed in the special container of the NMP, and the renal artery was connected to the pump for 24h of reperfusion. Each allograft received an arterial input and an individual venous outflow to have two separate perfusion circuits, one for the treated kidney and one for the control. The NMP was purchased from BMI (Virginia, USA) in collaboration with Virginia Tech University (Virginia, USA).

Perfusate analysis

For every experiment, 5 ml of perfusate from control and treated kidneys were collected at T0 (begin of perfusion in the NMP) and T24 (end of perfusion in the NMP, after 24h), and stored at -20 °C. Both NGAL and KIM1 concentrations on the in perfusate were analyzed at T0 and T24 through Enzyme-linked immunosorbent assay (ELISA). The NGAL kit were purchased from MyBioSource (MBS2702466), whereas the KIM-1 from Cloud-Clone corp. (PAA785Po01).

Immunohistochemical analysis

Biopsies collected at T24 were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, sliced into 5-mm sections and fixed onto polylysine-coated slides. Tissue sections were deparaffinized in xylene, hydrated gradually through graded alcohols, and then stained with hematoxylin and eosin by standard procedures.

In situ DNA 3-end labelling

Apoptosis was qualitatively identified in the testes using an in-situ thymidine deoxyribose-mediated deoxy-UTP nick end labelling (TUNEL) DNA 3-end labeling kit (Apoptag plus peroxidase in situ apoptosis detection kit, Merk Millipore). Paraffin sections were rehydrated through an alcohol series. The permeability of the cell membranes was increased by incubating the sections in 400 µg proteinase K (Roche Molecular Biochemicals) in 200 ml PBS for 15 min. Endogenous peroxidase activity was inhibited by quenching the samples for 5 min in 5% hydrogen peroxide. DNA fragmentation was identified by applying terminal transferase enzyme with digoxigenin-labelled nucleotides to the samples and incubating for 1 h under coverslips. Antidigoxigenin antibody was used to recognize the digoxigenin-labelled nucleotide chains attached to the 3-ends of sample DNA. A colour reaction was produced with diaminobenzidine in the presence of 0.03% hydrogen peroxide. The tissue sections were counterstained with hematoxylin. The apoptotic index was calculated as the ratio of apoptotic cells to total cells in each field, using five random fields.

8. RESULTS

Isolated mitochondria resulted respiration competent, capable of ATP production, and can be internalize by plated cells.

Prior to the assessment of the functional effect of AMT, the biological activity of isolated mitochondria obtained from renal proximal tubular cells, together with the evaluation of their internalization, was demonstrated *in vitro*. The augment of luminescence observed in the luciferin-oxyluciferin reaction, indicated that isolated mitochondria resuspended in respiration Buffer (RB) were capable of ATP production (fig. 12 A). Notably, increasing the amount of mitochondria resulted in the linear growths of luminescence (RLU). A further demonstration of the biological activity of isolated mitochondria was obtained by dyeing them with MitoTracker red CMXRos, as its accumulation depends upon the mitochondrial membrane potential. Red-florescent isolated mitochondria were optically detected, demonstrating the presence of a respiration-competent membrane potential (fig. 12 B). The internalization of mitochondria was demonstrated through optical visualization and flow cytometry. Mitochondria of human renal proximal tubular cells (HK-2) were labelled with GFP. GFP-labelled mitochondria were, then, isolated, and co-incubated with healthy HK-2 cells. The presence of GFP-labelled mitochondria was detected in plated cells at least three hours after co-incubation (fig. 12 C). To further confirm the obtained results, isolated mitochondria were labelled with MitoTracker red, and then co-incubated with HK-2. After co-

incubation, cells were detached, and the fluorescence emission was analysed. Compared with negative control, HK-2 treated with isolated mitochondria exhibited a red-fluorescent emission, confirming the optical evaluation (fig. 13). Taking together, these results confirmed that, after isolation, mitochondria from renal proximal tubular cells are biologically active and capable of ATP production. Furthermore, isolated mitochondria can be actively absorbed by renal proximal tubular cells.

AMT significantly enhances the proliferative capacity and decrease cytotoxicity *in vitro,* in primary ciPTEC cells subjected to I/R damage.

After the assessment of mitochondria internalization, we focused on the possible protective effect exerted by AMT in an *in vitro* model of ischemia/reperfusion injury (IRI), using conditionally immortalized human proximal tubular (ciPTEC). Primary ciPTEC cells were exposed to IRI, and then subjected to AMT (fig. 14). Isolated mitochondria were obtained from healthy primary ciPTEC cells. AMT treatment resulted capable to revert the proliferative impairment generated by IRI, as demonstrated by the increase of ATP production (fig. 15 A) and by the BRDU proliferation assay (fig. 15 B). Furthermore, the decrease of cell cytotoxicity was demonstrated by the significant improvement of accumulation and cleavage of GF-AFC peptide in primary ciPTEC underwent to AMT (fig. 15 C).

AMT is able to revert the metabolic impairment generated after I/R exposure *in vitro*.

To better characterize the mechanism of AMT recovery, we focused on the specific effect exerted on mitochondria and mitochondrial-related pattern. AMT treatment on primary ciPTEC significantly decreased the overall oxidative stress, as demonstrated by the decrease of ROS generation (fig. 16 A) and TBARS production (fig. 16 B). Coherently, AMT diminished the reduction of mitochondrial membrane potential after IRI (fig. 16 C), as demonstrated by JC-1 analysis. Furthermore, AMT was able to revert the detrimental effect on the expression of important enzymes correlated with TCA cycles (citrate synthase, alpha-ketoglutarate, succinate, malate) and oxidative phosphorylation (fig. 17 A-D), suggesting an improvement of mitochondrial function. Interestingly, we detected a significant recovery of glutaminase expression, an enzyme highly present in tubular kidney cells correlate with ammonium ions secretion and activation of metabolism (fig. 18 A). Coherently with the aforementioned results, an augmented ETC activity (fig. 18 B) and ATP production (fig 18 C) was detected in primary ciPTEC after IRI and AMT treatment. Taking together, these results demonstrated that AMT treatment is able to significantly revert the proliferative impairment and partially restore the mitochondrial network homeostasis generated in an *in vitro* model of renal tubular IRI.

AMT treatment partially reverted the deleterious effect of ischemia in an *ex vivo* porcine model of organ preservation.

To further confirm the obtained results, AMT was tested in porcine an *ex vivo* of RT preservation. Both kidneys of male Yorkshire pigs were collected and exposed to warm ischemia, in order to mimic the ECD situation. Then, one of the kidneys was perfused for 30' with isolated mitochondria in RB, whereas the other with RB alone, as control. Mitochondria were obtained from a skeletal muscle section of the same pig. After the treatment, both the kidneys were connected to a normothermic machine perfusion (NMP) for 24h. Immediately before the AMT treatment and 24h after perfusion in the NMP a biopsy of both the kidneys were collected for further analysis. In order to evaluate alterations in the presence of damage markers, samples of the perfusate were collected at the beginning (T0) and at the end (T24) of the perfusion. The schematic representation of the experimental project is depicted in figure 19.

First, the presence of damage markers KIM-1 and NGAL in the perfusate were analysed through ELISA. At T0 we did not observe any difference in expression of KIM-1 and NGAL between treated and controls kidneys. As expected, at T24, both control and treated kidneys showed an increase in the expression of these markers; however, the augmentation is less marked in organs subjected to AMT, as visible in figure 20 A, B. The difference of expression between control and treated kidneys falls to be significative, mostly due the low number of replicate and the physiologically high variability of this model. Nevertheless, a marked trend between the two condition is clearly visible. After the assessment of damage

markers in the perfusate, we focused on the analysis of the tissue homeostasis in the biopsies at T24. The histological evaluation of the H&E staining revealed the presence macroscopic damage in both the treated and the control kidneys (fig. 21 A, B). Interestingly, the TUNEL analysis highlighted a significant difference in the presence of apoptotic cells between treated and control kidneys (fig 21 C). considering these findings, the differences in both the expression of kidneys-associate damage markers and the number of apoptotic cells between treated and control samples suggests a biological protective effect exerted by isolated mitochondria, confirming the *in vitro* results.

Figures

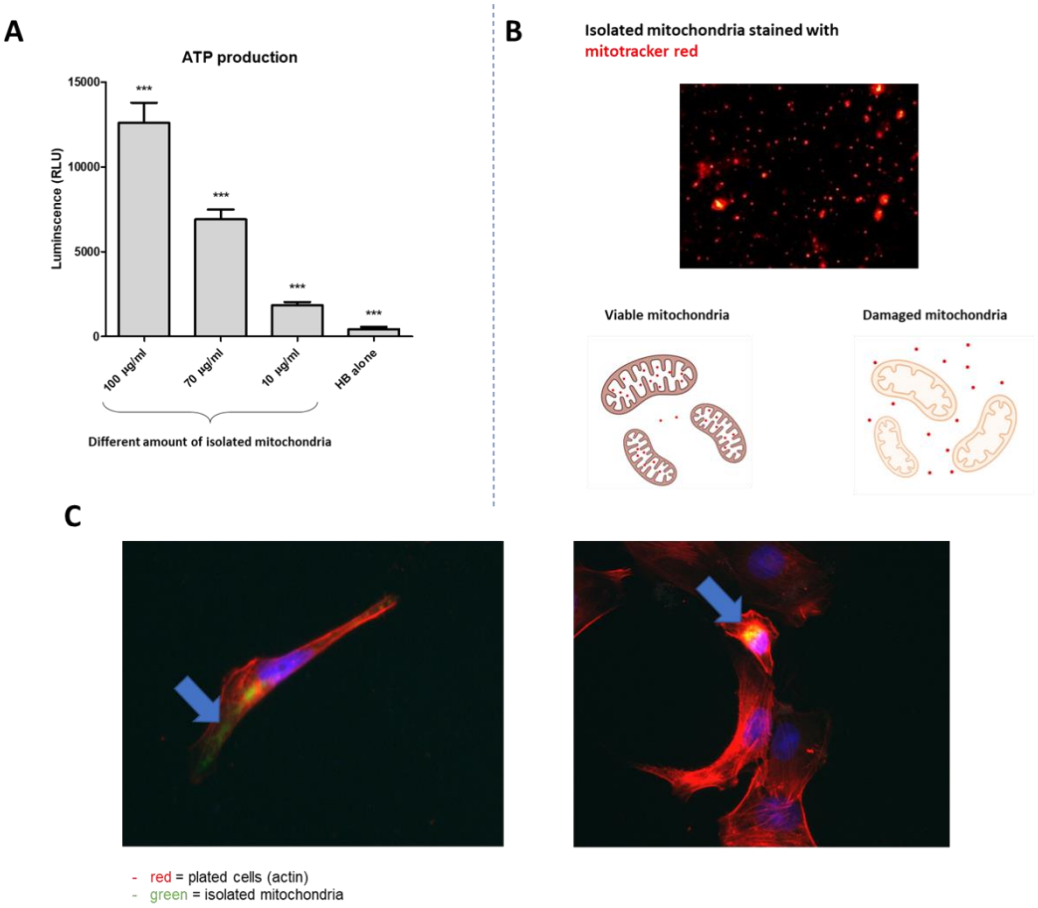


Figure 12. A. Isolated mitochondria were resuspended in RB at different concentrations (100 µg/ml, 70 µg/ml, 10 µg/ml, and buffer alone (HB)). The increase of luminescence caused by luciferin-oxyluciferin reaction indicates that the increase mitochondria concentration resulted in a linear increase in ATP concentration. n=3 **B.** Up, representative image of isolated mitochondria labelled with mitotracker red. Down, mitotracker is absorbed from viable mitochondria. **C.** Representative of mitochondria internalization. GFP-labelled mitochondria are absorbed by cultured cells. Actin is labelled in red.

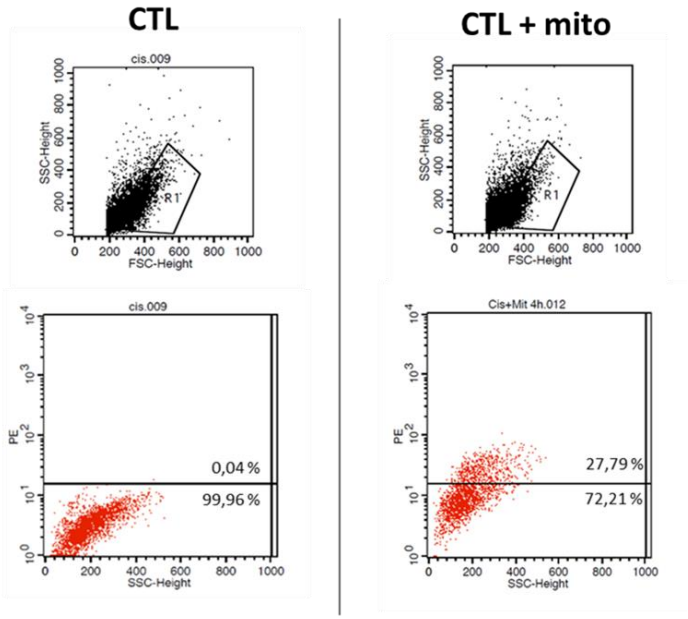


Figure 13. Isolated mitochondria were labelled with mitotracker red and then co-incubated with plated HK-2 cells. Cells were then washed three time with PBS, detached and analyzed through flow cytometry. Cell co-incubated with red-mitochondria exhibited red fluorescence, proving the absorption of isolated mitochondria.

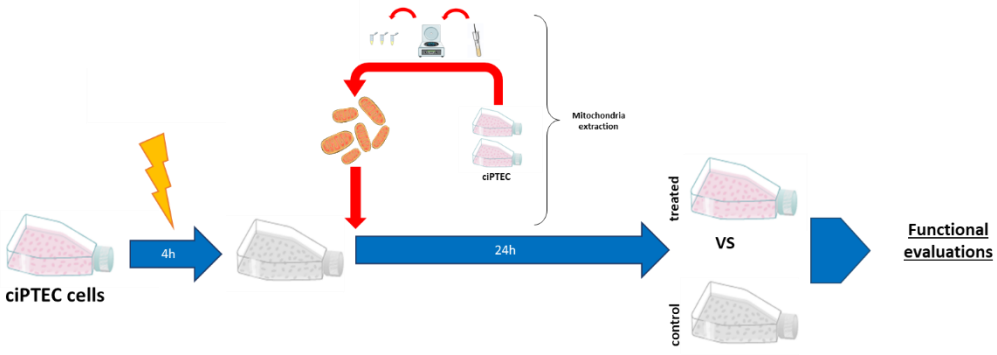


Figure 14. Experimental scheme of the *in vitro* experiments. ciPTEC cells were subjected to IRI, and then co-incubated with mitochondria isolated from healthy ciPTECs for 24h. after that, cells were washed three times rinsed with normal medium for 72h.

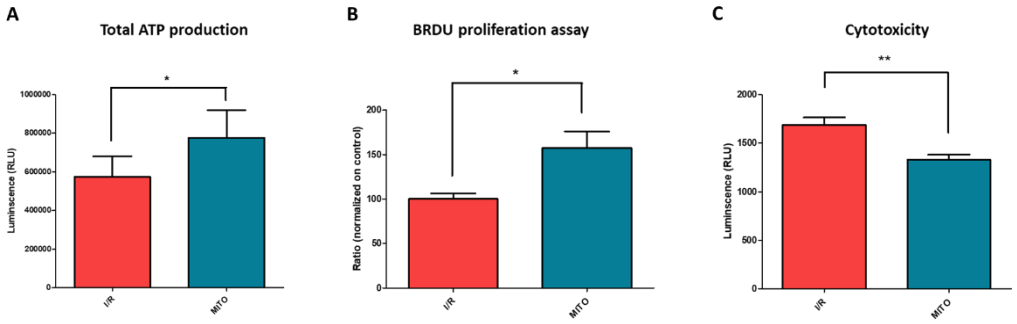


Figure 15. A. Quantification of total ATP production. AMT was able to increase total ATP production after IRI damage. n=4. **B.** AMT increased the proliferation capacity of ischemic ciPTEC cells plated in 96 well/plates in equal number. n=4. **C.** ciPTECs treated with mitochondria showed a reduced cytotoxicity compared to controls. n=6. For all these experiments, ciPTEC cells were cultured in 96 well/plate in equal number in each condition and subjected to IRI. Then were treated with mitochondria in RB or RB alone.

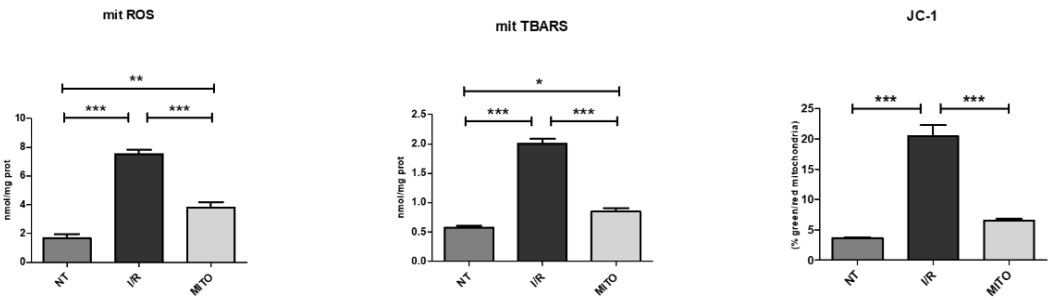


Figure 16. A. AMT is able to reduce the ROS augmentation subsequent to IRI. **B.** ROS decrease is further confirmed from the decreasing of TBARS after AMT. **C.** JC-1 analysis showed the restoration of mitochondrial membrane potential exerted from AMT treatment.

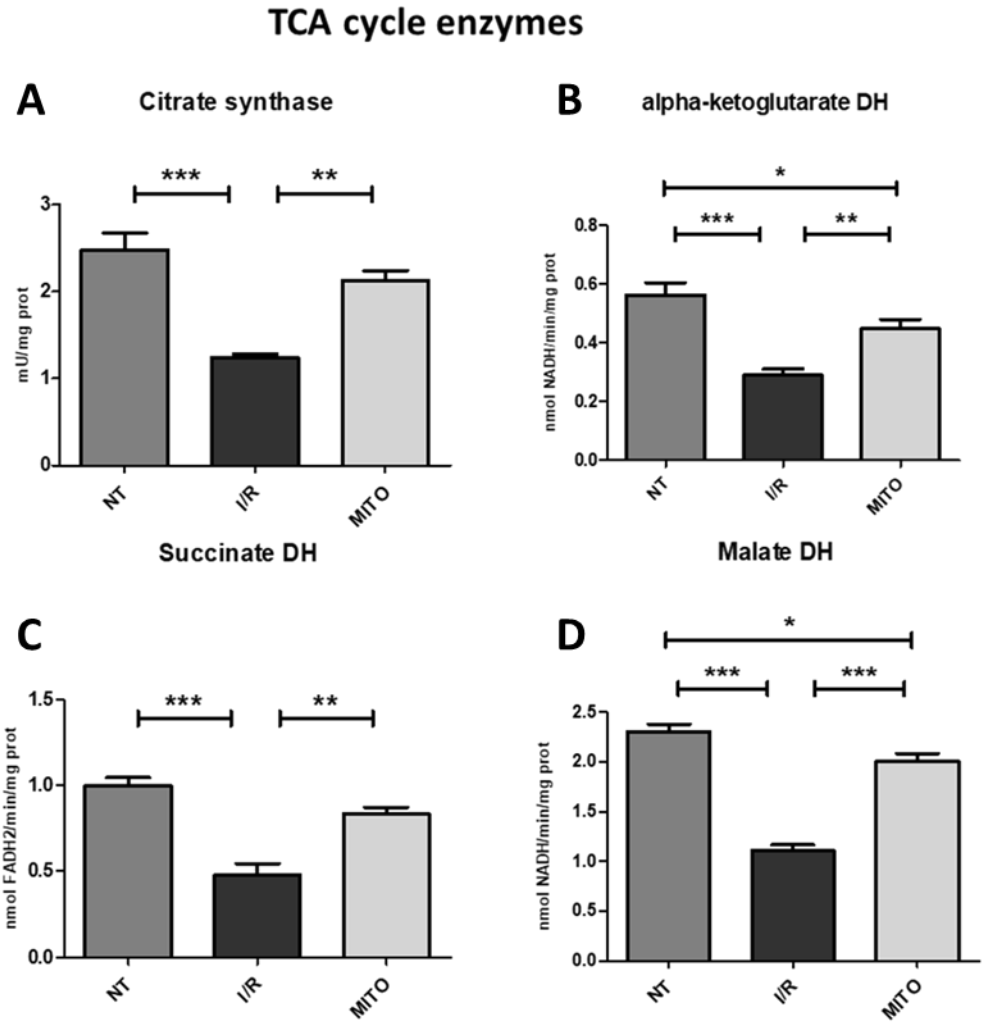


Figure 17. AMT treatment restored the activity of key TCA cycle-related enzymes after IRI treatment. **A.** Citrate synthase is commonly used as a quantitative enzyme marker for the presence of intact mitochondria. **B.** catalyzes the overall conversion of 2-oxoglutarate (alpha-ketoglutarate) to succinyl-CoA and CO2 during the citric acid cycle. **C, D.** succinate is oxidized to fumarate by succinate DH, followed by malate DH.

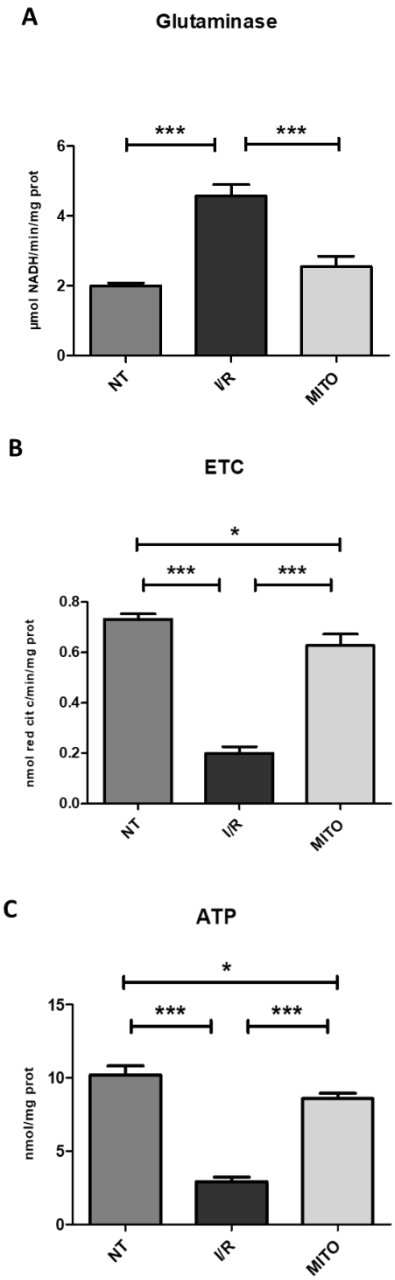


Figure 18. A. AMT was able to lower the activity of glutaminase expression, an enzyme highly present in tubular kidney cells correlate with ammonium ions secretion and activation of metabolism. **B, C.** Electron transport chain (ETC) and total ATP concentration were significantly increased after AMT.

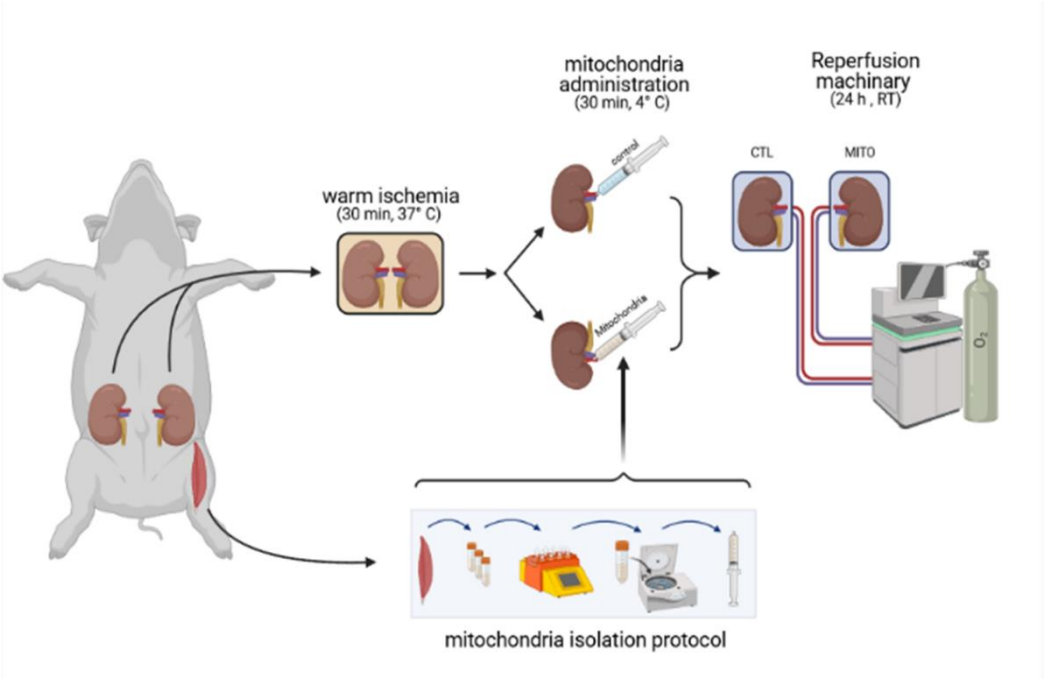


Figure 19. Graphic visualization of the *ex vivo* experimental setting. Both kidneys were collected immediately after pig sacrifice, and subjected to warm ischemia for 30 min. Then, one kidney was treated with isolated mitochondria whereas the other used as control. After 30 minutes, both kidneys were connected to NMP for 24h.

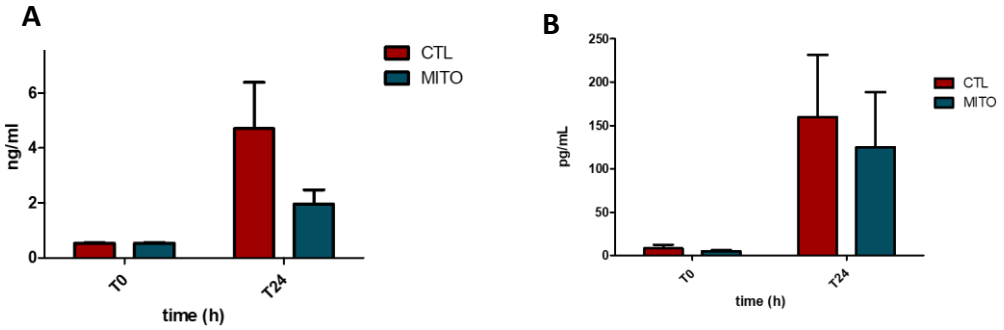


Figure 20. A. Level of NGAL in the perfusate in control kidney (red) and mitochondria-treated kidney (blue), at T0 and T24. **B.** KIM-1 expression in the perfusate in control kidney (red) and mitochondria-treated kidney (blue), at T0 and T24.

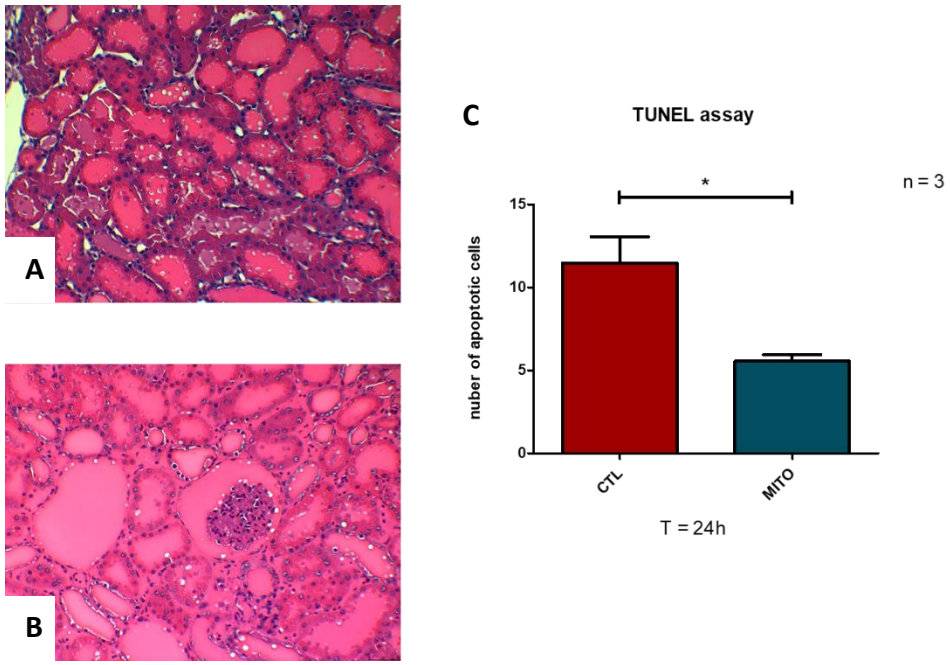


Figure 21. histological evaluation of the H&E staining in control (**A**) and treated (**B**) revealed the presence macroscopic damage in both the treated and the control kidneys. **C.** TUNEL assay revealed the decrease of apoptosis induced by IRI damage after AMT treatment.

9. DISCUSSION

Organ transplantation is victim of its own success. In view of its excellent results, the demand for transplantable organs is escalating while the supply has reached a plateau. Consequently, waiting times and patient's mortality on the waiting list are increasing dramatically [10]. Yet, the majority of the organs that are procured for transplant purposes are discarded [11]. In the case of RT, which is the most frequently transplanted organ worldwide, one third of the organs that are procured from deceased donors are not used, mainly for severe IRI [12]. It is intuitive that the identification of strategies to render these kidneys transplantable represents one of the most urgent needs of modern transplantation. The overarching goal of our research is therefore to provide evidence of the efficacy of AMT in treating IRI in the setting of RT. This need is even more urgent if we consider the data relative to the CKD pandemic and its costs. In fact, 10% of the world population is affected by CKD; in the US, one out of 7 individuals. In 2010, 2.62 million people received dialysis worldwide but the need for dialysis was projected to double by 2030. This means that the number of patients that will eventually need a new kidney will increase exponentially in the next decade. Moreover, transplant is more cost-effective than alternative renal replacement strategies like dialysis [12]. In 2015, in the US, Medicare expenditures on chronic and end-stage kidney disease were more than 64 billion and 34 billion dollars, respectively. Much of the expenditure, morbidity and mortality previously attributed to diabetes and hypertension are now attributable to kidney disease and its complications [13]. Traditionally, organs

procured from deceased donors for transplant purposes have been stored at 2-4°C in order to reduce anaerobic metabolism and mitigate the cascade of event triggered by ischemia that may culminate in cellular death [14]. Unfortunately, cold storage does not completely halt the above sequence of events. In fact, studies involving cold storage of human proximal tubular epithelial cells showed that cell death was predominantly from necrosis, with signs of mitochondrial swelling and membrane disruption. Upon rewarming and reperfusion, there is a switch to apoptosis of the epithelial cells. Additional evidence suggests that with as little as 2 hours of cold ischemia time, there is an increase in mitochondrial permeability transition pores, with translocation of cytochrome C and subsequent activation of the Bcl-2 and caspase-3 pathways. As the mitochondrial respiratory complexes begin to fail during ischemia, ROS are formed and this is undesirable because a sudden burst in ROS production will also occur after reperfusion in the recipient, so overwhelming the physiological antioxidant mechanisms [14]. Herein, we propose AMT as mechanism of amelioration of marginal renal allografts that are subjected to normothermic machine perfusion (NMP). We are doing so for two reasons. First, robust data show that NMP is superior to cold storage and has the dual potential of improving organ function post transplantation, while allowing for accurate functional assessment in a near-physiologic state, helping to improve kidney utilization and outcomes from marginal graft. Second, it has been recently shown that AMT prolongs cold ischemia time to 29 hours in a murine model of heart transplantation, significantly enhances graft function, and decreases graft tissue injury [11].

In the first part of the project, we evaluated the possible protective effect of AMT *in vitro*, on human renal proximal tubular cells. To work efficiently, tubular cells require high amounts of ATP; thus, the disruption of the mitochondria networking subsequent to IRI have a great influence of tubular cells homeostasis. The cell line we used were the ciPTEC cells, conditionally immortalized cells that can be easily expanded and, at the same time, be switched to primary cells for experiments. Firstly, we showed that AMT on ciPTEC underwent to IRI increased the proliferation capacity and decreased the intracellular cytotoxicity compared to control, similarly to previous result from Konari et al [6]. The second step was to deeper investigate the possible protective effect of AMT on the mitochondrial network. We detected a significant decrease in mitochondrial membrane potential alteration following AMT, together with the reduction of ROS and Thiobarbituric Acid Reactive Substances (TBARS), byproducts of ROS augmentation. At the same time, the assessment of the activity of mitochondrial-related enzymes showed the significant increase in the activity of TCA cycle related enzymes (citrate synthase, alpha-ketoglutarate, succinate, and malate) as well as enzymes involved in oxidative phosphorylation. Coherently with these observations, we measured an increased intracellular ATP concentration in AMT-treated cells. Taking together, this data demonstrated that AMT is able to revert the detrimental effect of IRI *in vitro*, rescuing the proliferative capacity of cell and diminishing the cytotoxicity. AMT is, moreover, able to restore the partially disrupted mitochondrial homeostasis, increasing the activity of mitochondrial-related enzymes, restoring the mitochondrial membrane potential, and increasing the ATP production *in vitro*.

In the second part of our work, conducted in cooperation with Wake Forest University (Wiston-Salem, NC, USA), we tested AMT treatment in an *ex vivo* preclinical model of RT preservation. The kidneys of three Yorkshire pigs were subjected to warm ischemia; then, one was treated with isolated mitochondria from skeletal muscle, whereas the other was injected with RB alone and used as control. Our findings suggest that AMT is able to partially revert the tissue damage affected by IRI. In fact, the presence of KIM-1 and NGAL in the perfusate resulted diminished, while the number of apoptotic cells in the control was significantly higher than treated. Other experiments are planned to further confirm the protective effect of AMT *ex vivo*. In particular, we planned to deeper analyze the presence of indicative proteins and markers in the perfusate using the RAMAN spectroscopy, together with the investigation of the protein expression through RNA seq of biopsies.

References

[1] J. Wang *et al.*, "Stem cell-derived mitochondria transplantation: A novel strategy and the challenges for the treatment of tissue injury," *Stem Cell Res. Ther.*, vol. 9, no. 1, pp. 1–10, 2018, doi: 10.1186/s13287-018-0832-2.

[2] D. Liu *et al.*, "Intercellular mitochondrial transfer as a means of tissue revitalization," *Signal Transduction and Targeted Therapy*, vol. 6, no. 1. Springer Nature, pp. 1–18, Dec. 01, 2021, doi: 10.1038/s41392-020-00440-z.

[3] L. Peruzzotti-Jametti *et al.*, "Neural stem cells traffic functional mitochondria via extracellular vesicles to correct mitochondrial dysfunction in target cells," *bioRxiv*, p. 2020.01.29.923441, Jan. 2020, doi: 10.1101/2020.01.29.923441.

[4] A. A *et al.*, "Human mesenchymal stem cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer," *Stem Cells*, vol. 29, no. 5, pp. 812–824, 2011, doi: 10.1002/STEM.632.

[5] H. C. Lin, S. Y. Liu, H. S. Lai, and I. R. Lai, "Isolated mitochondria infusion mitigates ischemia-reperfusion injury of the liver in rats," *Shock*, vol. 39, no. 3, pp. 304–310, 2013, doi: 10.1097/SHK.0b013e318283035f.

[6] N. Konari, K. Nagaishi, S. Kikuchi, and M. Fujimiya, "Mitochondria transfer from mesenchymal stem cells structurally and functionally repairs renal proximal tubular epithelial cells in diabetic nephropathy in vivo," *Sci. Rep.*, vol. 9, no. 1, pp. 1–14, 2019, doi: 10.1038/s41598-019-40163-y.

[7] J. D. McCully, D. B. Cowan, C. A. Pacak, I. K. Toumpoulis, H. Dayalan, and S. Levitsky, "Injection of isolated mitochondria during early reperfusion for cardioprotection," *Am. J. Physiol. - Hear. Circ. Physiol.*, vol. 296, no. 1, pp. 94–105, 2009, doi: 10.1152/ajpheart.00567.2008.

[8] J. D. McCully, S. Levitsky, P. J. del Nido, and D. B. Cowan, "Mitochondrial transplantation for therapeutic use," *Clin. Transl. Med.*, vol. 5, no. 1, 2016, doi: 10.1186/s40169-016-0095-4.

[9] P. JM, P. CA, K. H, M. AA, C. DB, and M. JD, "Rapid isolation and purification of mitochondria for transplantation by tissue dissociation and differential filtration," *J. Vis. Exp.*, no. 91, Jun. 2014, doi: 10.3791/51682.

[10] G. Orlando, S. Soker, and R. J. Stratta, "Organ bioengineering and regeneration as the new holy grail for organ transplantation," *Annals of Surgery*, vol. 258, no. 2. Ann Surg, pp. 221–232, Aug. 2013, doi: 10.1097/SLA.0b013e31829c79cf.

[11] S. Giwa *et al.*, "The promise of organ and tissue preservation to transform medicine," *Nature Biotechnology*, vol. 35, no. 6. Nat Biotechnol, pp. 530–542, Jun. 07, 2017, doi: 10.1038/nbt.3889.

[12] G. Orlando *et al.*, "Discarded human kidneys as a source of ECM scaffold for kidney regeneration technologies," *Biomaterials*, vol. 34, no. 24, pp. 5915–5925, Aug. 2013, doi: 10.1016/j.biomaterials.2013.04.033.

[13] C. W. Yang *et al.*, "Global case studies for chronic kidney disease/end-stage kidney disease care," *Kidney International Supplements*, vol. 10, no. 1. Kidney Int Suppl (2011), pp. e24–e48, Mar. 01, 2020, doi: 10.1016/j.kisu.2019.11.010.

[14] G. Orlando and S. Keshavjee, "Organ repair and regeneration preserving organs in the regenerative medicine era."

Acknowledgement

Firstly, I would like to thank my supervisor Prof. Benedetta Bussolati for the continuous assistance and suggestions during the three years of my PhD.

Secondly, I would like to thank Linda, Sofia, Lola, Roberta, Sarah, Renata, Fabio, Natalia all the other members of lab 10 and 11 for all the days together and for all the hints.

My sincere gratitude goes to prof. Giuseppe Orlando, and all the member of his lab for the amazing support during my months of work in Wake Forest University. In particular, I would like to thank Giuseppe, Marie Chantal and Emilio for the amazing hospitality from the first to the last day in Winston-Salem.

Last but not least I would like to thank my family and Francesca to be always on my side during these three years.