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Review Article

Quality Control Parameters for Mitochondria Transplant in Cardiac Tissue

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Abstract

Transplant of mitochondria for cardioprotection has been shown to be efficacious in vitro and in vivo in animal models. The transition from animal to human models requires that effective quality control be used in the isolation and verification of mitochondrial viability and function. Herein, we describe standard procedures that can be used to isolate mitochondria and assess their quality and function. These procedures include hemocytometry, Coulter Counting, EM, fluorescent staining, respiration analysis and ATP assays. These quality control parameters for mitochondria transplant in cardiac and other tissue will ensure a high yield of isolated mitochondria from suitable tissue sources, and will ensure that the isolated mitochondria are viable, respiration competent and free from contamination.

ABBREVIATIONS

ATP; adenosine triphosphate, ADP; adenosine diphosphate, DNA; deoxyribonucleic acid, EM: transmission electron microscopy, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, LDH; lactate dehydrogenase, OCR; oxygen consumption rate, RCI; respiratory control index , RNA; ribonucleic acid.

INTRODUCTION

Mitochondria function/ dysfunction have been shown to play an important role in the modulation of cellular function and response in the myocardium [1-5]. In previous studies we and others have demonstrated that myocardial ischemia detrimentally alters mitochondrial structure, mitochondrial volume, mitochondrial calcium accumulation, mitochondrial complex activity, mitochondrial oxygen consumption, mitochondrial high energy synthesis and the mitochondrial mediated intrinsic apoptotic pathway [1-5].These events occur during ischemia and extend into reperfusion to severely compromise post-ischemic functional recovery and cellular viability, significantly increasing myocardial necrosis and apoptosis [1-5]. We hypothesized that the transplantation of mitochondria into the myocardial ischemic zone during early reperfusion would augment or replace the function of mitochondria damaged during ischemia and would allow for enhanced post-ischemic functional recovery and cellular viability.

Our investigations have demonstrated that transplantation of viable, structurally intact, respiration competent, autogeneic mitochondria, isolated from the patient's own body from remote skeletal tissue unaffected by ischemia, into the ischemic zone of the myocardium during early reperfusion significantly decreases myonecrosis, significantly decreases reactive oxygen species, significantly enhances ATP stores following ischemia and significantly enhances post-ischemic functional recovery [2,3]. To ensure that mitochondria transplant is efficacious, quality control parameters for tissue procurement, isolation and determination of purity and viability have been established. We present these quality control parameters in brief below.

Tissue sources and dissection

Establishing sterile aseptic operating techniques for tissue dissection and isolation is essential to enable mitochondria transplant. All equipment must be sterilized and aseptic and sterile operating and isolation techniques must be adhered to. In our studies we have isolated mitochondria from non-ischemic pectoralis major muscle as it is available at the mini-thoracotomy incision site. Alternative tissue sources may be used; however, the general techniques are similar. Prior to incision and dissection, the incision site is shaved and prepped with Betadine and 70% isopropyl alcohol, each applied in triplicate and patted dry with sterile gauze pads and then draped. An intercostal incision is made, the pectoralis major is located and two biopsy specimens are retrieved using a number 6 biopsy punch. These biopsy specimens provide >1 x 10^9 mitochondria [2,3]. The biopsy specimens are then placed in a pre-cooled sterile tube containing sterile PBS and kept on ice prior to isolation of mitochondria.

Mitochondrial isolation

The major consideration for clinical utilization must be purity and time for isolation. All solutions and equipment are sterile and all techniques must be performed in a sterile environment. We suggest the use of a laminar flow hood such as that used in tissue culture. Mitochondrial isolation must be performed immediately for use in mitochondria transplant. Long time storage > 2 hours decreases mitochondrial function and mitochondrial uptake [2,3,21] . Non-viable mitochondria, previously frozen mitochondria, mitochondrial fractions (proteins, complex I-V), mitochondrial DNA and RNA, and exogenous ATP or ADP do not provide cardioprotection [2,3].

The majority of mitochondrial methods involve tissue homogenization and differential centrifugation. A comprehensive review of these methods has been previously published [6,7]. The number of homogenization and centrifugation steps varies among protocols but all include repetitive centrifugation. These repetitive centrifugation steps increase the time for mitochondrial isolation to >90 min. and ultimately reduce viability [3,6-7,11-14]. In addition, manual homogenization can cause mitochondrial damage and inconsistent results if not properly controlled [6,8].

To allow for standardized mitochondria isolation we have replaced manual homogenization and the inherent variability therein with standardized automated tissue dissociation and homogenization using a commercial dissociator [15]. The major benefits of this protocol are that standardized tissue dissociation allows for uniform and consistent homogenization of tissue that is not easily achieved with manual homogenization. Standardized tissue dissociation of standardized tissue samples (two #6 biopsies) provides consistent homogenization and digestion. In addition, rather than differential centrifugation we recommend the use of differential filtration that eliminates time consuming and repetitive centrifugation steps allowing for more rapid isolation of highly purified, viable and respiration competent mitochondria [15]. The use of standardized automated tissue dissociation and homogenization and differential filtration provides purified, viable, respiration competent mitochondria in < 30 min., a time frame within the majority of surgical procedures.

Mitochondrial purity

stablishing the purity of isolated mitochondria is paramount. The isolated mitochondria must be substantially free of cytosolic, nuclear or microsomal components. Common preparation contaminants include fragments from endoplasmic reticulum, endosomes, golgi apparatus, nucleus, and cytosol [16]. Enzymatic analysis of mitochondria isolates for cytosolic and cytoplasmic contaminant markers glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) and microsome contaminate markers 5´nucleotidase and glucose-6-phosphatase should be performed regularly. Western blot analysis should be used to confirm that protein components from all mitochondrial compartments are present and that contaminant markers are absent from mitochondrial preparations [9,14,17].

We also recommend that Southern blot analysis for housekeeping genes actin and GAPDH as markers of nuclear DNA contamination should be performed with appropriate controls. Evidence of nuclear DNA contamination indicates contamination and may induce possible immune or auto-immune response if transplanted with mitochondria.

Mitochondrial number

The number of isolated mitochondria can be determined in many ways. The easiest way is by hemocytometry [2,3]. This technique is cost effective but is variable and user dependant. We have found that when using hemocytometry to estimate the number of isolated mitochondria, the mitochondrial number is underestimated one to 3 fold.In additions, as hemocytometry is observer dependent, the estimate of mitochondria number can be inconsistent [15]. We recommend that for more consistent results all estimations by hemocytometry be made by a single observer.

An alternative to hemocytometry is to estimate mitochondria number based on mitochondrial protein content. This method is performed using serial dilutions of an estimated mitochondrial number. The disadvantages are that both algebraic and observer dependant bias may be present.

For optimal estimation of mitochondria number we recommend the use of the Beckman Multisizer 4e Coulter Counter or a similar device. Particle counters like the Multisizer 4e are commonly used by researchers and have proven to be effective in the clinic [18-22]. The estimation of mitochondria number is automated and results are not observer dependent. In our studies we have observed that particle size counting is useful in assessing both number and purity. Particle counting also provides for estimate of isolated mitochondria diameter and allows for another means of comparison to other reports [15,23].

Mitochondrial viability

Mitochondria viability is most rapidly and easily assessed using mitochondrial fluorescent probes [2,3,24,25]. In our studies we have used MitoTracker and MitoFluorprobes in combination [2]. MitoTracker Orange CMTMRos (Invitrogen,Carlsbad CA) is an orange-fluorescent dye that stains mitochondria and its accumulation is dependent upon mitochondrial membrane potential. The use of this probe allows for identification of viable, respiration competent mitochondria. MitoFluor Green labels all mitochondria independent of membrane potential. The use of both MitoTracker Orange CMTMRos and MitoFluor Green with overlay allows for the identification of all viable mitochondria within a preparation as the orange and green overlay appear as yellow [2,3]. Appropriate wavelengths must be chosen to measure auto-fluorescence and background fluorescence, using unstained mitochondria [2,3].

Mitochondrial function

Mitochondrial function can be examined by polarographic or spectrophotometric techniques. Clark electrode measurements are the best and most reliable means of assessing mitochondrial function. Oxygen consumption rate (OCR) can be measured by Clark type electrodes, including Oxytherm (Hansatech Instruments Ltd, Norfolk, UK), XF^{e} analyzer (Seahorse Bioscience Inc, Massachusetts, USA), Oxygraph-2K (OROBOROS instruments Corp, Innsbruck, AUT).

Mitochondrial function at complex I of the electron transport chain (ETC) is assessed in energized mitochondria using malate and glutamate, energy substrates specific to complex I. Complex II function is assessed using succinate an energy substrate specific to complex II and rotenone an inhibitor of complex I. State 2 mitochondrial oxygen consumption is measured prior to the addition of ADP which then initiates state 3 mitochondrial oxygen consumption. Once state 3 has proceeded for two minutes, state 4 mitochondrial oxygen consumption is initiated by blocking the ATP synthase of complex V with addition of oligomycin [2,25]. The respiratory control index (RCI) is calculated as the ratio of the state 3/state 4 mitochondrial oxygen consumption [2,25].

In addition to mitochondria OCR we have found that the measurement of mitochondrial complex activity is needed to confirm mitochondrial function. In general we measure complex I,II,III,IV and V activity. All reactions are performed by spectrophotometric analysis [26-29]. The determination of mitochondrial complex activity allows for the identification of any impairment in mitochondrial function that may detrimentally affect short- and long-term function.

ATP content is another way mitochondrial function can be measured. In our research we have used standardized techniques, however, we have found that the ATP luminescence assay (ATPlite Luminescence ATP Detection Assay System, Perkin Elmer) provides a quick and reproducible measurement of mitochondrial ATP production [15,30,31]. The ATPlite assay system provides similar results as those obtained by Clark electrode and therefore is compatible with previous data analysis [32-34].

DISCUSSION AND CONCLUSION

Isolated mitochondria are suitable for transplant if they are viable, respiration competent and free of contamination. For eventual transition to clinical application, it is important to ensure that mitochondrial preparations are isolated under sterile, aseptic conditions [9,17]. All solutions are either autoclaved or filter sterilized and stored at -20°C prior to use. The use of stock solutions to prepare final isolation solutions is recommended.

It is important to note that the use of excess tissue does not significantly increase the number of mitochondria isolated. It has been our experience that increased starting tissue content leads to extended isolation time due to filter clogging and decreased viability of the isolated mitochondria. The use of two #6 biopsies provides approximately 1 x 10^9 mitochondria, more than sufficient for clinical application.

In our studies we have used 1 x 10^6 to 1 x 10^9 mitochondria for injection into the myocardium. The absolute number of mitochondria that can be used for transplant has not been established. Our studies to date indicate that 1 x 10^7 provides optimal results and that increased concentration does not enhance efficacy [2,3]. It should be noted that we have not observed any conduction abnormalities either by serial electrocardiography or by optical mapping with increased mitochondrial transplant concentrations.

Determination of viability is important for efficacy. Confirmation of viability and function should be performed following each isolation and whenever tissue sources are changed or isolation solutions are changed.

We recommend that all quality control assays be performed following the change of any solution used in the isolation procedure. Following initial confirmation, mitochondrial fluorescent probes should be used to test an aliquot following each isolation to confirm mitochondrial viability, at a minimum. Analysis using mitochondrial fluorescent probes can be performed rapidly and does not significantly extend the preparation time for isolated mitochondria. In our experience labelling with mitochondrial fluorescent probes can be done in 5-10 min. with analysis being performed in 5-10 min. The results from mitochondrial fluorescent probes have not differed from that obtained by ATP luminescent or by polarographic or spectrophotometric techniques requiring greater time to perform.

We have found that transmission electron microscopy (EM) is also a valuable method to identify preparation contaminants and to assess mitochondrial structure and purity [2,3,6,8,9,14]. While the use of EM is not appropriate for rapid evaluation, representative samples should be stored in appropriate buffer to allow for future analysis for purity and contamination.

Western blot analysis and enzymatic analysis of cytosolic and cytoplasmic contaminant markers and additional studies to confirm mitochondrial complex activity are suggested at appropriate intervals. These assays will confirm mitochondrial, purity, integrity, function and viability.

In our studies we have used all the assays described above to confirm that isolated mitochondria are pure, viable and respiration competent. Familiarity with the assays lessens time for preparation and evaluation. While some of the assays are simple and can be performed rapidly, the elimination of any of the remaining assays is not recommended.

In conclusion, we present an outline for quality control parameters for mitochondria transplant in cardiac and other tissue that will ensure a high yield of isolated mitochondria from suitable tissue sources, and ensure that the isolated mitochondria are viable, respiration competent and free from contamination.

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