A Modified Method For Mitochondrial Purification And DNA Isolation From Muscles

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Abstract:

Normally, the muscles are rich in mitochondria, but in myopathic patients, the muscles are degenerated, and low mitochondrial content is land marked. In the present modification a small piece of muscle biopsy (0.5-0.8gram) was taken twice at a time from each patient (12 patients with primary muscular dystrophy). The first biopsy was processed by the standard method, while the other was subjected to the modified method. In the latter method, the muscle biopsy was first minced and then digested with the enzyme trypsin III at a concentration of 0.35mg / gram for 10 minutes. In the standard method, the enzyme concentration was 0.25mg /gram, and the incubation time was 30 minutes. Therefore, some time saving was reached. But, the more important finding was highly enriched mitochondrial pellet, as well as, no contamination with other cellular components was observed. This modification has led to isolate a pure mtDNA, which was not contaminated with nuclear DNA.

Introduction:

Mitochondria are double membrane organelles devoted to energy production (1), so its responsible for the production of energy in eukaryotic cell in the form of Adenine triphosphate (ATP) by oxidative phosphorylation, a process that requires the orchestrated actions of five respiratory enzyme complex located in mitochondrial inner membrane (2). Dysfunctional mitochondria are involved in pathogenesis of disorders devoted mitochondrial diseases (3) that include a wide variety of degenerative diseases, aging and cancer. 40 years ago mitochondria was firstly implicated to cause these diseases when a patient with hyper metabolism whose skeletal muscles contained large number of abnormal mitochondria, that contain mutated mitochondrial Deoxyribonuclic acid (mtDNA) (4,5).

There are many methods for isolation of mitochondria and mtDAN like ultracentrifugation, involving cesium chloride gradients (6), or by using polymerase chain reaction (PCR) technique (7). However, expansive equipment and relatively long experimental time are required for these methods. The mitochondria and their genetic material (mitochondrial DNA; mtDNA) are now reaching great advances, especially in researches related to mitochondrial disorders, such as myopathies. Therefore, a development of methods which yield high content of mitochondria and a pure mtDNA from muscles is highly required, especially in patients with myopathic atrophy. The present investigation aimed to reach such goal.

This study presents more fast and simple method for Isolation of mitochondria from muscle of patients with myopathy, also using alkaline denaturation method which is used for plasmid isolation (8) to isolate mtDNA from these patients.

Materials and methods:

Muscle biopsy & pathological analysis

12 patients with primary muscular dystrophy (MD) were studied. *Vastus medials* muscle biopsies were done under local anesthesia (2% Lidociane hydrochloride) for histochemistry & mitochondrial Isolation.

Histochemical processing includes staining for Nicotine Adenine Dehydrogenase NADH), Succinate Dehydrogenase (SDH). Gomori -trichrome (T.C)& Eosin -heamatoxylin (E&H)was done as well.

Small pieces of muscle [0.5 - 0.8 gm] obtained for mitochondria, mitoblast and their DNA studies.

Preparation of mitochondria & mitoblast:

A modificated procedure of that described by (9) & (10) was used. It was prepared from 0.5 -0.8 gm of muscle biopsy. All steps were carried out at ice bucket temperature in buffer [0.35M sucrose; 0.01M Na_2 - EDTA; 0.035M Tris - base : PH 7.8], the muscle was minced once using tissue grinder and resuspended again in 30 ml of the same buffer with 0.35 mg /g Trypsin III (sigma).

The minced tissue was stirred on ice for 10min then rinsed 5-6 series of the same buffer to get rid of the excess trypsin III. Then homogenized by hand (4-5 passes), adjusted to 10 % w/v homogenate.

The final homogenate was centrifuged at 3000 g for 5 min, the resultant supernatant was recentrifuged at 8500g for 10 min, the mitochondrial pellet was resuspended in about 0.5 ml of the same buffer, then divided into two groups [each of 0.25ml], the first one used to prepare mitoblast using the method described by (11).

Mt DNA Isolation (or Extraction):

Mt DNA were prepared from mitochondrial pellet & mitoblast that isolated above using Alkaline denaturation method described by (8) with some modification [using the pellet of mitochondria directly in 10 mM Tris - EDTA buffer and 0.18N NaOH containing 1% sodium dodocyl sulfute (SDS) with out using any kind of proteases].

Result & Discussion:

Most mitochondria are between 0.5-1.0 micrometer in diameter and 7 Micro meter in length although the size and shape can vary, and are often called the powerhouses of the cell ,because it convert the chemical energy of glucose product to the chemical energy of ATP molecules, (1) by many enzymes make mitochondria too delicate and damaged very quickly .

In this method the resultant mitochondria were so pure and obtaind in shorter time than the other methods (6,9) by raising the trypsin concentration from 0.25 mg\g to 0.35 mg /g , which digest the muscles faster because Trypsin splits bonds involving the carboxyl group of the basic amino acids lysine and arginine .

This enhancement made the muscels digestion faster , which is good as the shorter the time of isolation the better will be the chance of survival of mitochondria as well in this method, a decrease in the a mount of muscle needed in comparison to other's, with less hazards and discomfort for the patients who complain from muscle weakness and atrophy.

Mitoblaste was prepared by using digitonine, which digest the first membrane of mitochondria and the other organelle like nucleus , This step was done to be sure there is no other cell organelle reside with mitoblaste ,then we sure that DNA isolated from mitochondria only, and not from other cell organelle

The isolated mt DNA in this method was so pure as compared with that from mitoblasts . The purity might be attributed to that mitochondria contain no nuclei .

Other advantages of this new method is by using the alkaline denaturation method so we can isolate close circular covalent mtDNA from the nuclear one. Also the method was relatively cheap and less coast .

The DNA isolated here can be used in Polymerase chain reaction (PCR) method more easily in order to get large a mount of mtDNA for research purposes. The electrophoresis result usually 3 bands, (according to the physical from of mtDNA) but here (fig.1) It was found a new 4^{th} band seen in line (1, 2, 11).

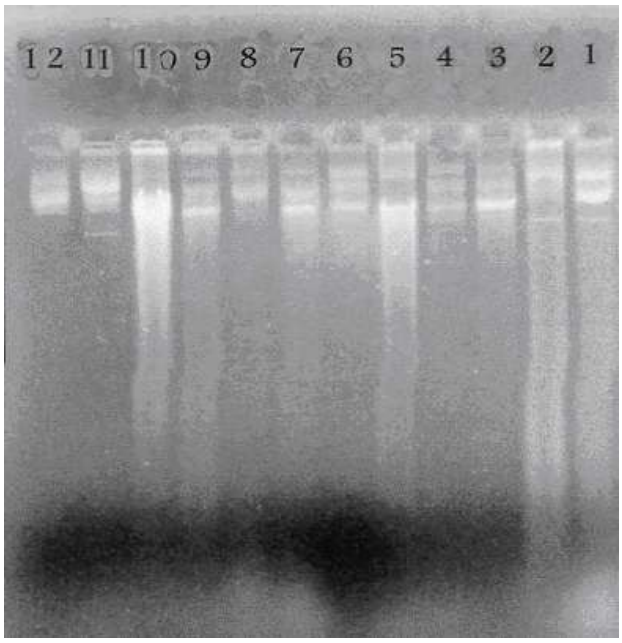

Fig: (1) Represent extraction DNA from myopathy patients: Line (1, 2, 11).new 4^{th} band.Line 5, 10 low concentration of mtDNA, with one band only and the smear indicate nDNA. Other lines ; could be normal mt DNA (three bands refer to three physical forms of mt DNA).

This mean a new segment of mtDNA of less molecular weight since it pass more rapidly down, this might be due to deletion happened in mtDNA in long stands diseased patient which will cause an abnormal enzymes system in the mitochondria, which will lead to more deterioration in the pathology of the disease for more details look for (3,12). Line 5, 10 (fig-1) which indicate low concentration of mtDNA , with one band only, can be explained due to the presence of other rare band with low concentration, these results were from patients with mitochondrial myophathy and Ducheene muscular dystrophy (long standing), might be due to end stage muscle , few lakes of muscle fibers only.

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طريقة محورة لتنقية المايتوكوندريا واستخلاص الDNA من العضلات

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الملخص:

عادة تكون العضلات غنية بالمايتوكوندريا باستثناء مرضى الاعتلال العضلي الذين تعاني عضلاتهم من نقص في المايتوكوندريا نتيجة لتحللها. اعتمادا على طريقة استخلاص DNA المحورة الحالية حيث يمكن اخذ قطعة صغيرة من العضلة المصابة (٠,٥-٠,٨)غم من (١٢) مريض ذوي الشذوذ العضلي الابتدائي وبمكررين لكل عينة. احدها عوملت بطريقة الاستخلاص القياسية و الأخرى بالطريقة المحورة التي تركز التعديل فيها على زيادة تركيز إنزيم التربسين III ليصل إلى ٠,٣٥ ملغم/غم وخفض فترة الحضن لتصل إلى ١٠ دقائق في حين كانت الطريقة الأساسية تعتمد على تركيز الإنزيم ٠,٢٥ ملغم/غم وفترة حضن تصل إلى ٣٠ دقيقة، مما أفضى إلى الحصول على كمية كبيرة من المايتوكوندريا فضلا عن نقاوتها من مكونات الأخرى وهذا أدى إلى استخلاص كمية اكبر من الDNA المايتوكونيدري غير ملوث بالـ DNA النووي ، فضلا عن اختصار بعض الوقت المهم في هكذا تحليلات.