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# RAPID PROTOCOL FOR PREPARING HUMAN ENDOMETRIAL MITOCHONDRIA FOR TRANSPLANTATION

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**Abstract:** Most of the applied methods for isolation of intact mitochondria are time consuming, required special equipment and are based on expensive commercial kits. Here, we have developed a simple, fast, cost-effective, and labor-efficient protocol for preparing endometrial functionally active mitochondria from primary cell culture. Primary endometrial cells were grown on microplates, detached with 2.9 mM EDTA (pH 6.14) added directly to wells containing cell culture medium (RPMI 1640 and 10% FBS) and harvested mechanically with cell scrapers, centrifuged at 600 g for 10 minutes, homogenized by using syringes with 27G and 30G needles. The mitochondria were isolated by differential two-step centrifugation (1300 g for 5 minutes and 12000 g for 15 minutes). All procedures were done on ice and the centrifugation steps were carried out at $4^0$C in STE buffer with 10 μl/ml protease inhibitor. The quality of the obtained mitochondria have been analyzed using flow cytometric assessment of mitochondrial membrane potential after staining with dye JC-1 and luminescence analysis of adenosine triphosphate (ATP) measured by ATP determination kit. This isolation protocol requires about 40 minutes and yields approximately $2 \times 10^7$ viable mitochondria from $1 \times 10^9$ culture cells with good membrane potential and high ATP content. The developed procedure bypasses washing steps, differential filtration, reducing the cell and mitochondrial loss that occurs in standard multistep protocols. The obtained viable mitochondria were intended for respiration studies and following *in vivo* transplantation as an alternative treatment of women with endometrial pathologies and recurrent implantation failure (RIF).

## INTRODUCTION

Mitochondria are essential organelles that control the cell life cycle and participate in key metabolic reactions, synthesize majority of the ATP and regulate a large number of signalling pathways (Frezza *et al.,* 2007; Bratic and

Larsson, 2014). Mitochondria damage, caused by stress usually has detrimental effects on cellular and metabolic processes (Chan, 2006). In addition, the number of mitochondria as well as their functionality decreases with age (Conley *et al.,* 2000; Johannsen *et al.,* 2012). As a consequence, an increase in reactive oxygen species (ROS) production and mutations in mtDNA have been observed (Payne and Chinnery, 2015). These factors are prerequisites for age-related diseases (Bratic and Larsson, 2014.). Recent studies have shown that mitochondrial dysfunction accelerates DNA damage, which leads to genetic instability and drives tumorigenesis (Sung *et al.,* 2010). Inappropriate function of mitochondria could also cause dynamic cytotoxic changes in reproductive tissues, which could be a reason for infertility or early miscarriage (Schatten *et al.,* 2014; Cecchino *et al.,* 2018).

Nowadays, it is known that mitochondrial transfer as a clinical procedure could provide beneficial effects for tissue repairing procedures (Elliott *et al.,* 2012; McCully *et al.,* 2016). This method has an enormous potential for application in treatment of infertility problems caused by endometrial dysfunction and pathology. Therefore, isolation stages are of crucial importance in the process of obtaining viable and intact mitochondria, applicable for these purposes (McCully *et al.,* 2016). Many mitochondrial isolation methods have been developed, aiming to find the most appropriate treatment procedures (Bensley and Hoerr, 1934; Claude, 1946; Hogeboom *et al.,* 1948.; Ernster, and Schtaz, 1981). The majority of them are based on incorporate homogenization and differential centrifugation (Graham *et al.,* 2001; Frezza *et al.,* 2007; Pallotti and Lenaz, 2007; Gostimskaya and Galkin, 2009; Wieckowski *et al.,* 2009; Fernández-Vizarra *et al.,* 2010; Schmitt et al., 2013). The number of homogenization and centrifugation repetitive steps varies among protocols and could increase the time for mitochondrial isolation and reduce viability. In addition, particular stages of the isolation process such as manual homogenization can also cause mitochondrial damage (Preble *et al.,* 2014).

The purpose of our study was to develop and describe a simple, fast, cost-effective, and labor-efficient protocol for preparing endometrial functionally active mitochondria from cell culture.

## MATERIALS AND METHODS

### Protocol description

### Cell culture

Human endometrial cells (mixed culture of stromal and epithelial cells) were seeded and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% Penicilin-Streptomycin-Amphotericin, 1% L-glutamine at 37°C in 5% $CO_2$. Cells were grown as a monolayer in appropriate plates until they reach at least 80% confluence.

**Mitochondria isolation**

2.9 mM EDTA (pH 6.14) was added directly to the wells and cells were incubated at room temperature for 5 minutes. The wells were scraped to remove the adherent cells from the bottom surface and the media containing the cell suspension was aspirated and transferred to individual 15 ml conical tubes, placed on ice. Then, the tubes were centrifuged for 10 minutes at 600 x g, 4°C. The supernatant was aspirated and the pellet was resuspended in 1 ml STE buffer (250 mM sucrose, 5 mM Tris, and 2 mM EDTA; pH 7.4) with 10 µl/ml protease inhibitor cocktail (PI 78415; Thermo Fisher Scientific, USA) and 0.5% human serum albumin (HSA) (Vitrolife, Sweden). Then, the cell suspension was homogenized using syringes with 27G and 30G needles. The obtained solution was drawn into a 3 ml syringe using a 27 gauge needle and it was expelled back into the conical tube on ice with the same needle. This step was repeated for a total of 30 times. After that, the solution was drawn into a 3 ml syringe using a 30 gauge needle and expelled it back into the conical tube on ice with the same needle. The step was repeated 30 times. The homogenized solution was transferred to a 1.5 tube and then, it was centrifuged for 5 minutes at 1300 g, 4°C. The supernatant was transferred in a new 1.5 ml tube and the described centrifugation step was repeated 2 times. After that, the supernatant containing the mitochondria was transferred to a new 1.5 tube and centrifuged for 15 minutes at 12000 g, 4°C. The supernatant was aspirated and the pellet was combined with 100 µl STE buffer and immediately placed on ice.

**Verification of the isolation method and assessment of mitochondrial functionality**

**Patients/Samples**

32 biopsy samples taken from 32 women with normal fertility during the mid-luteal phase of the menstrual cycle were used for cell cultivation. The obtained endometrial cell cultures were used for optimization and verification of the mitochondria isolation protocol. All patients who participated in this study signed an informed consent that was approved by the Institution Review Board (IRB) of Nadezhda Woman's Health Clinic, Sofia, Bulgaria.

**Flow Cytometry Analysis**

All flow cytometry experiments were performed on a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA). The fluorescent probe was purchased from Invitrogen/Molecular Probes (Eugene, OR, USA). Mitochondrial membrane potential was evaluated with 2 µM JC1 (catalog No. T3168; ex488, em530/590). Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). Flow cytometry was used also to determine the number of isolated mitochondria.

**Luciferin-luciferase assay for adenosine triphosphate (ATP)**

The ATP Determination Kit (A22066, Invitrogen) was employed for the luciferin-luciferase assay using the vendor's protocol. To each well in a 96-well plate, the kit reaction buffer was added to the samples or standard solution provided by the kit at room temperature. The samples and standards were read immediately on a microplate reader DTX 880 (Beckman Coultier, Fullerton, CA) for measurement of the ATP content of intact mitochondria.

**Quantification of mitochondrial protein content**

Quantification of mitochondrial protein content was determined through colorimetric assay (Bradford, 1976). The Bradford assay utilizes a colorimetric change of Coomassie Brilliant Blue G-250 from 465 nm (unbound) to 595 nm when bound to protein, with protein concentration being proportional to the absorption at 595 nm with reference to a standard curve of known concentrations.

**Statistical Analysis**

Descriptive statistical analyses were carried out with SPSS version 21 (IBM, Armonk, New York, USA). All values are shown as mean ± standard deviation. The number of observations refers to the number of preparations (independent experiments) analyzed.

## RESULTS AND DISCUSSION

The procedure steps in the isolation of mitochondria using the described method are shown in Figure 1. Total procedure time is approximately 40 min. Cultured primary endometrial cells were confluent in day 1-3 (**Fig. 2**). Measurement of total protein content (from 109 cultured cells) using Bradford assay revealed that the described isolation method provides mitochondria with 450±36 µg protein content (**Table 1**). Mitochondria viability and functionality was determined by JC1 and ATP measurement. The obtained results showed that large percentage of the isolated mitochondria maintained the membrane potential (**Fig. 3**) and had relatively high levels of ATP (**Table 1**). The estimated ATP content was 10.67± 4.38 nmol/mg mitochondrial protein.

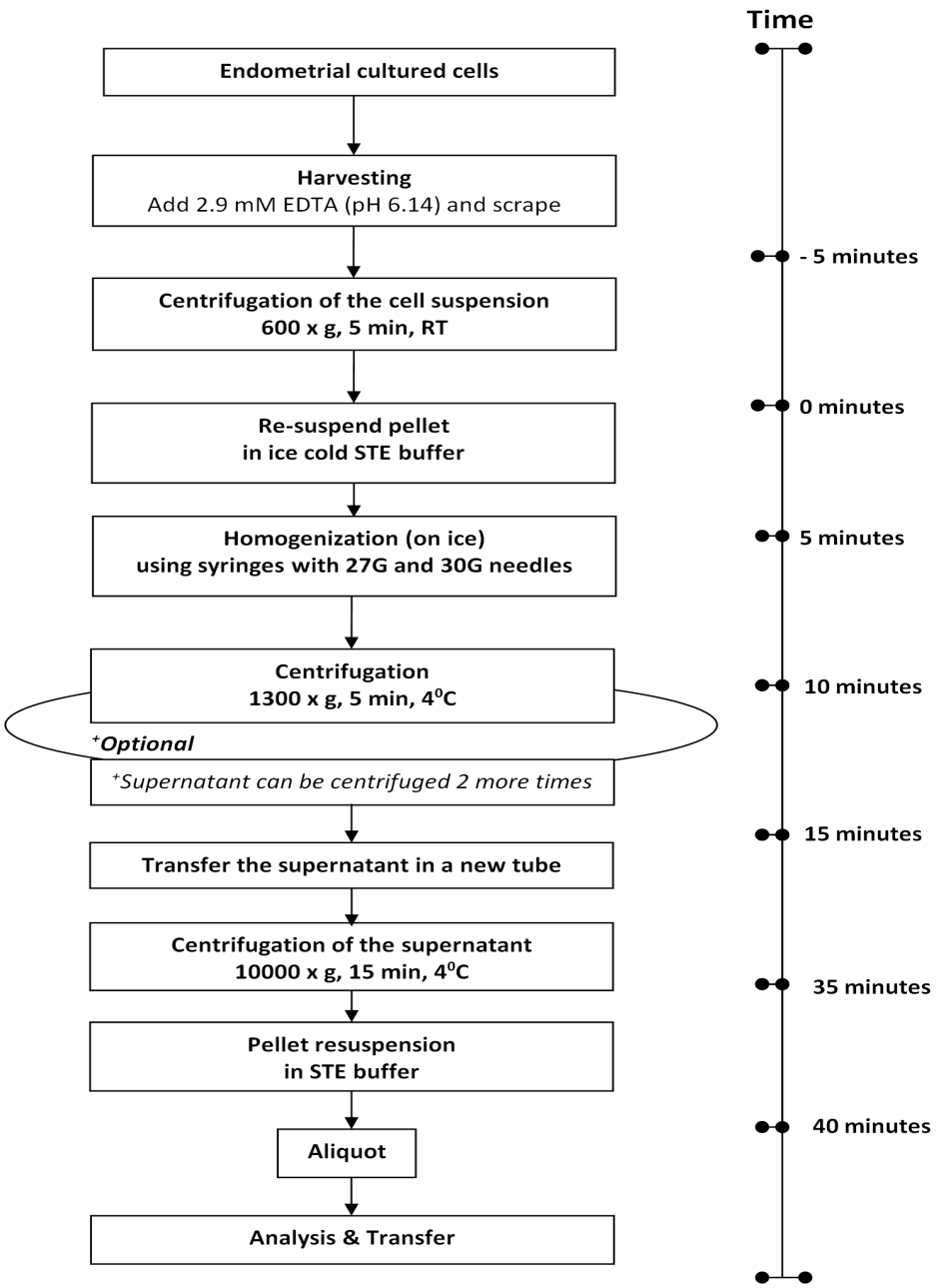


**Fig. 1.** General flowchart for isolating mitochondria from cultured endometrial cells. Note that all steps of isolation, except centrifugation, were performed on ice or at a temperature below $4^0$C.

**Table 1.** Yield characteristics and functional parameters of the isolated mitochondria using the described isolation protocol.

| Parameter | Value |
|---|---|
| Used number of culture cells, N | $(1\pm0.2)x10^9$ |
| Mitochondria yield, N | $(2\pm0.4)x10^7$ |
| Mitochondria protein content (from $10^9$ cultured cells), µg | 450±36 |
| ATP content, nmol/mg mitochondrial protein | 10.67± 4.38 |

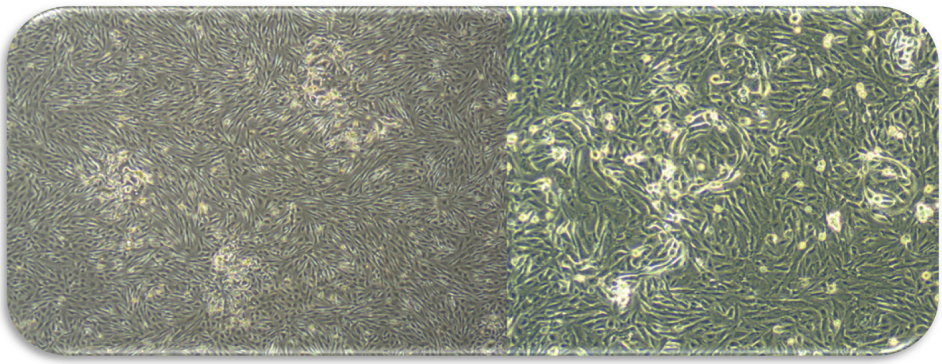

**Fig. 2.** Bright field microscopy of endometrial cell populations (mixed cultures) used for the mitochondria isolation procedure. Confluent monolayer from endometrial stromal and endometrial epithelial cells

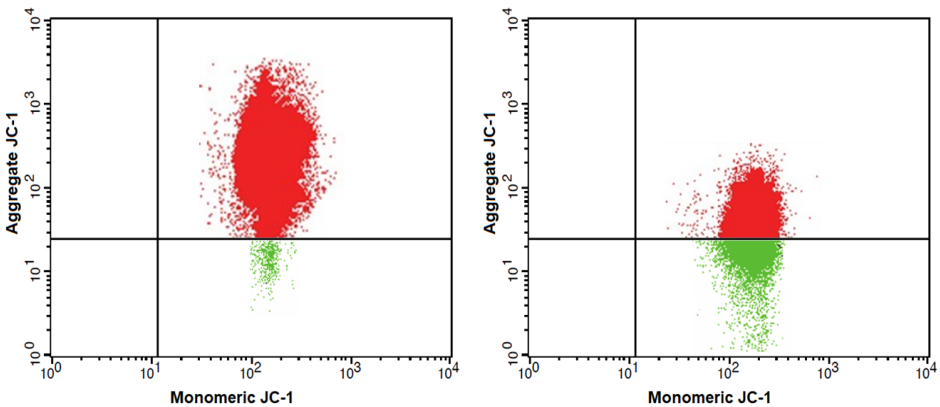


**Fig. 3.** Shift of fluorescence emission from red to green of the JC-1 Fluorescent Probe indicating loss of mitochondrial membrane potential with time - 30 minutes of incubation (left panel) vs. 8 hours of incubation (right panel).

To assess the levels of loss of mitochondrial membrane potential with time after the isolation procedure, the measurements from several time-points were compared (**Fig. 4**). During the first four hours JC1 fluorescence intensity was relatively stable. Eight hours after the performed mitochondria isolation procedure, a dramatic decrease in the ratio of red-green fluorescence intensity was observed, indicating rapid depolarization of mitochondrial membranes (**Fig. 3**). Mitochondria showed a sustained loss (greater than six-fold decrease) of membrane potential for 24 hours after the end of the isolation procedure.

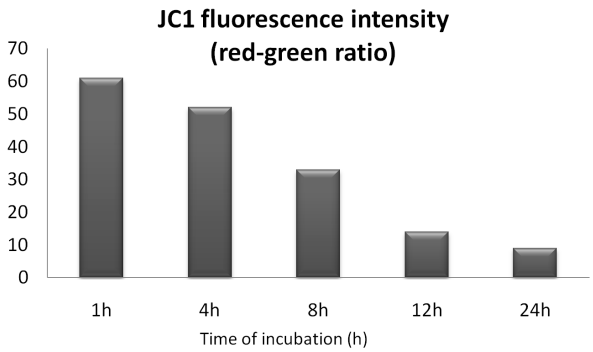


**Fig. 4.** Normal mitochondrial potential, measured after staining with JC-1, decreases with time of incubation after the performed isolation procedure. Mean changes in ΔΨm were measured spectrofluorometrically.

Despite the existence of many procedures for isolation and *in vitro* analysis of human mitochondria, it is of crucial importance to adopt the protocol for specific tissue type in order to receive intact and functionally active mitochondria. A new cost-effective and time-efficient method to isolate relatively pure mitochondria is essential for different applications such as transplantation procedures. Since isolated mitochondria are targeted to transfer into new cells *in vitro* or *in vivo*, new methods have been developed – microinjection, peptide mediated mitochondrial delivery (PMD) and liposome mediated transfer (Irwin *et al.,* 1999). Kit-based methods also usually give a good proportion of active mitochondria for transplantation (Hartwig et al., 2009; Elliott *et al.,* 2012). However, their main disadvantage is that they are relatively expensive (Elliott et al., 2012). In a study which compared kit-based and manual methods for isolation of mitochondria from the human liver cell line (HepG2), the proportion of intact and active mitochondria was significantly higher in the kit-based methods (Azimzadeh et al., 2016). However, the applied manual mitochondrial isolation method yielded greater number of mitochondria. Compared to these results, we succeed to achieve

significantly better mitochondrial yield and quality, optimising the conditions for isolation from endometrial cell cultures (**Table 1**).

For *in vitro* protein synthesis purposes and *in vivo* transplantation, it is preferable to apply methods that emphasizes the preparation of intact mitochondria rather than the total yield (Pallotti *et al.,* 2007). Some of the existing manual procedures for mitochondrial isolation are based on the application of discontinuous Percoll® gradient (Sims *et al.,* 1990). However, similarly to our isolation procedure, most of the published manual protocols apply methods, consisting of sequential steps of homogenization, washing and centrifugation between 600 g and 12,000 g (Hosseini *et al.,* 2013; Mashayekhi *et al.,* 2014).

An important advantage of our manual isolation protocol is that it allows acquisition of viable mitochondria in less than 40 minutes and yields approximately $2x10^7$ viable mitochondria from $1x10^9$ culture cells with good membrane potential and high ATP content. Another positive feature of our procedure is that it bypasses washing, differential filtration, reducing the cell and mitochondrial loss that occurs in standard multistep protocols. Taking into consideration the cost-effectiveness, our protocol as well as many developed methods aims to simplify the procedure of mitochondria isolation and purification. A recently described method for isolation of mitochondria from PBMCs for proteomics analysis didn't use expensive equipment such as ultracentrifuge (Pooreydy *et al.,* 2013). Other authors succeed to exclude homogenisation step in the protocol for mitochondrial isolation from tissue (Gross *et al.,* 2011). In our protocol, we also reduced the number of standard isolation steps and the required equipment.

The homogenization step is a critical point for the final yield of mitochondrial preparations. Moreover, decreased level of mechanical stress is essential for successful isolation of intact and fully active mitochondria. For this reason the homogenisation was done by using syringes with 27G and 30G needles instead of using homogenizer. An alternative method for mechanical homogenization of cultured cells applied by other authors is disruption by nitrogen cavitation (Hunter and Commerford, 1961). It was used to isolate mitochondria from primary cultures of neurons and astrocytes (Kristian *et al.,* 2006). The advantage of this procedure is that it also limits the shear stress due to mechanical forces.

Human cell cultures are considered as a good source of mitochondria in relation to their easy identification, cultivation and availability (Frezza *et al.,* 2007). In our study we used human endometrial cell cultures for mitochondria isolation. The advantages were that they can reach confluence for a short period of time (24÷48 hours) and could be cultured in standard conditions. These features additionally increase cost-effectiveness and yield levels of the developed method. Moreover, our isolation protocol could easily be modified for use with other cell lines or even primary tissues.

Previous studies have shown that the transplantation of healthy, intact and active mitochondria into cells containing defective ones could be used as a healing

procedure for a variety of diseases (Elliott *et al.,* 2012; Azimzadeh *et al.,* 2016). These procedures proved as an efficient treatment method in heart diseases, for enhancement of drug sensitivity in human breast cancer (Elliott *et al.,* 2012), for improvement of cell function in cells with DNA mutation (Chang *et al.,* 2013), Parkinson's disease (Chang *et al.,* 2016), liver ischemia (Lin *et al.,* 2013), and neurorecovery after stroke (Hayakawa *et al.,* 2016). Mitochondrial transfer has been also applied in assisted reproductive technologies and has the potential to provide an alternative technique for prevention of mtDNA disease and production of high quality oocytes (Reznichenko *et al.,* 2016). Many pathological conditions leading to lower fertility such as endometriosis are associated with mitochondrial dysfunction of particular cell types (Hsu *et al.,* 2014). The implementation of mitochondrial transfer in such cases could be considered as a perspective treatment procedure. The obtained viable mitochondria using our isolation protocol were intended for respiration studies and following *in vivo* transplantation as an alternative treatment of women with endometrial pathologies and recurrent implantation failure (RIF).

## CONCLUSION

The developed isolation procedure is an efficient method for obtaining viable and intact endometrial mitochondria for transplantation purposes.

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### DECLARATION OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

### AUTHORS CONTRIBUTION STATEMENT

D.P., G.S. and T.C. conceived the experiment; D.P. and I.S. conducted the experiment; D.P., T.C. and G.S analyzed the results. D.P. and I.S. wrote the main manuscript text and prepared tables and figures. T.C. and G.S. edited the manuscript and made its final revision. All authors critically reviewed and approved the final version of the manuscript.

## REFERENCES

1. Azimzadeh, P., Asadzadeh Aghdaei, H., Tarban, P., Akhondi, M.M., Shirazi, A., Khorram Khorshid, H.R. 2016. Comparison of three methods for mitochondria isolation from the human liver cell line (HepG2). *Gastroenterology and Hepatology From Bed to Bench,* 9 (2): 105-13.
2. Bensley, R.R., Hoerr, N. 1934. Studies on cell structure by freeze-drying method; preparation and properties of mitochondria. *The anatomical record,* 60(4): 449–455.

3. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2): 248–254.
4. Bratic, A., Larsson, N. 2014. The role of mitochondria in aging. *The Journal of Clinical Investigation,* 123 (3): 951-957.
5. Cecchino, G.N., Seli E., de Motta E.L.A., García-Velasco J.A. 2018. The role of mitochondrial activity in female fertility and assisted reproductive technologies: overview and current insights, *Reproductive Biomedicine Online,* 36 (6): 686-697
6. Chan, D.C. 2006. Mitochondria: Dynamic Organelles in Disease, Aging, and Development. *Cell*. 125 (7): 1241-1252.
7. Chang, J.C., Liu, K.H., Li, Y.C., Kou, S.J., Wei, Y.H., Chuang, C.S., Hsieh, M., Liu, C.S. 2013. Functional recovery of human cells harbouring the mitochondrial DNA mutation MERRF A8344G via peptide mediated mitochondrial delivery. *Neurosignals*. 21 (3-4):160-173.
8. Chang, J.C., Wu, S.L., Liu, K.H., Chen, Y.H., Chuang, C.S., Cheng, F.C., Su, H.L., Wei, Y.H., Kuo, S.J., Liu, C.S. 2016. Allogeneic/xenogeneic transplantation of peptidelabeled mitochondria in Parkinson's disease: restoration of mitochondria functions and attenuation of 6-hydroxydopamine–induced neurotoxicity. *Translational Research*, 170: 40-56.
9. Claude, A. 1946. Fractionation of mammalian liver cells by differential centrifugation II. Experimental procedures and results. *The Journal of Experimental Medicine*, 84 (1): 61–89.
10. Conley, K.E., Jubrias, S.A., Esselman, P.C. 2000. Oxidative capacity and ageing in human muscle. *J. Physiol.*, 526:203–210.
11. Elliott, R.L., Jiang, X.P., Head, J.F. 2012. Mitochondria organelle transplantation: introduction of normal epithelial mitochondria into human cancer cells inhibits proliferation and increases drug sensitivity. *Breast Cancer Research and Treatment*, 136 (2): 347-54.
12. Ernster, L., Schtaz, G. 1981. Mitochondria: a historical Review. *The Journal of Cell Biology,* 91 (3 Pt 2): 227s-255s.
13. Fernández-Vizarra, E., Ferrín, G., Pérez-Martos, A., Fernández-Silva, P., Zeviani, M., Enríquez, J.A. 2010. Isolation of mitochondria for biogenetical studies: An update. *Mitochondrion*. 10 (3): 253-262.
14. Frezza, C., Cipolat, S., Scorrano, L. 2007. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nature Protocols,* 2 (2): 287-295.
15. Gostimskaya, I., Galkin, A. 2010. Preparation of highly coupled rat heart mitochondria. *J Vis Exp.* (43): Pii:2202.
16. Graham, J.M. 2001. Isolation of mitochondria from tissues and cells by differential centrifugation. *Current Protocols in Cell Biology*, 3: Unit 3.3.
17. Gross, V.S, Greenberg, H.K., Baranov, S.V., Carlson, G.M., Stavrovskaya, I.G., Lazarev. A.V., Kristal, B.S. 2011. Isolation of functional mitochondria from rat kidney and skeletal muscle without manual homogenization. *Anal Biochem.* 418 (2): 213-223.

18. Hartwig, S., Feckler, C., Lehr, S., Wallbrecht, K., Wolgast, H., Muller-Wieland, D., *et al.* 2009. A critical comparison between two classical and a kit-based method for mitochondria isolation. *Proteomics*, 9 (11): 3209-14.
19. Hayakawa, K., Esposito, E., Wang, X., Terasaki, Y., Liu, Y., Xing, C., Ji, X., Lo, E.H. 2016. Transfer of mitochondria from astrocytes to neurons after stroke. *Nature*. 535 (7613): 551-555.
20. Hogeboom, H., Schneider, W.C., Palade, G.E. 1948. Cytochemical studies of mammalian tissues; isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. *The Journal of Biological Chemistry,* 172 (2): 619–635.
21. Hosseini, M.J., Shaki, F., Ghazi-Khansari, M., Pourahmad, J. 2013. Toxicity of vanadium on isolated rat liver mitochondria: a new mechanistic approach. *Metallomics*, 5 (2): 152-66.
22. Hsu, A.L., Townsend, P.M., Oehninger, S., and Castora, F.J. 2014. Endometriosis may be associated with mitochondrial dysfunction in cumulus cells from subjects undergoing *in vitro* fertilization-intracytoplasmic sperm injection, as reflected by decreased adenosine triphosphate production. *Fertil. Steril*,103 (2): 347–352
23. Hunter, M.J.. Commerford, S. L. 1961. Pressure homogenization of mammalian tissues. *Biochim.Biophys. Acta*, 47 (3): 580–586.
24. Irwin, M.H., Johnson, L.W., Pinkert, C.A.1999. Isolation and microinjection of somatic cell-derived mitochondria and germline heteroplasmy in transmitochondrial mice. *Transgenic Research,* 8 (2): 119-23.
25. Johannsen, D.L., Conley, K.E., Bajpeyi, S., Punyanitya, M., Gallagher, D., Zhang, Z., Covington, J., Smith, S.R., Ravussin, E. 2012. Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *J. Clin. Endocrinol. Metab*., 97:242–250.
26. Kristian, T., Hopkins, I. B., McKenna, M. C., and Fiskum, G. 2006. Isolation of mitochondria with high respiratory control from primary cultures of neurons and astrocytes using nitrogen cavitation. *J. Neurosci. Methods*, 152 (1-2): 136–143.
27. Lin, H. C., Liu, S. Y., Lai, H. S., Lai I. R. 2013. Isolated mitochondria infusion mitigates ischemia-reperfusion injury of the liver in rats. *Shock*. 39 (3): 304-310.
28. Mashayekhi, V., Eskandari, M.R., Kobarfard, F., Khajeamiri, A., Hosseini, M.J. 2014. Induction of mitochondrial permeability transition (MPT) pore opening and ROS formation as a mechanism for methamphetamine-induced mitochondrial toxicity. *Naunyn Schmiedebergs Arch Pharmacol*, 387 (1): 47-58.
29. McCully, J.D., Levitsky, S., del Nido, P.J. and Cowan, D.B. 2016. Mitochondrial transplantation for therapeutic use. *Clinical and Translational Medicine,* 5(16): 1-13
30. Pallotti, F., Lenaz, G. 2007. Isolation and subfractionation of mitochondria from animal cells and tissue culture lines. *Methods in Cell Biology,* 80: 3-44.
31. Pooreydy, B., Jafari, M., Tajik, F., Karimi, M., RezaeiTavirani, M., Ghassempour, A., et al. 2013. Organelle Isolation for Proteomics: Mitochondria from Peripheral Blood Mononuclear Cells. *J Paramed Sci,* 2013 (4): 78-86.
32. Preble, J.M., Pacak, C.A., Kondo, H., et al. 2014. Rapid isolation and purification of mitochondria for transplantation by tissue dissociation and differential filtration. *Journal of Visualized Experiments*, (91):e51682.

33. Reznichenko, S.A., Huyser, C., Pepper, M. 2016. Mitochondrial transfer: Implications for assisted reproductive technologies. *Applied & Translational Genomics.* 11 (2016): 40–47
34. Schatten, H., Sun, Q., and Prather, R. 2014. The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility. *Reprod. Biol. Endocrinol.,* 12: 111
35. Schmitt, S., et al. 2013. A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies. *Anal Biochem.* 443 (1): 66-74.
36. Sims, N.R. 1990. Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. *J Neurochem,* 55 (2): 698-707.
37. Sung, H.J., Wenzhe, M., Wang, P., Hynes, J., O'Riordan, T.C., Combs, C.A., McCoy, J.P., Bunz, F., Kang, J., Hwang, P.M. 2010. Mitochondrial respiration protects against from oxygen-associated DNA damage. *Nature Communications*, 1: 5.
38. Wieckowski, M.R., Giorgi, C., Lebiedzinska, M., Duszynski, J., Pinton, P. 2009. Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. *Nature Protocols*, 4 (11): 1582-1590.