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Postmortem mitochondrial membrane permeability transition assessment of apoptotic cell death in brain and liver of insulin resistant, ovariectomised rats

Ebenezer I.O. Ajayi [a,c,*], Olorunfemi R. Molehin [b], Alex E. Iyoha [a], Divya S.P. Tallapragada [c], Omotade I. Oloyede [b], Kulbhushan B. Tikoo [c]

[a] Biochemistry Department,Faculty of Basic and Applied Sciences,College of Science, Engineering and Technology, Osun State University, P.M.B. 4494, Osogbo, Nigeria
[b] Department of Biochemistry, Faculty of Science, Ekiti State University Ado-Ekiti, P.M.B. 5363, Ado Ekiti 360001, Nigeria
[c] Pharmacology/Toxicology Department, National Institute of Pharmaceutical Education and Research, S. A. S. Nagar (Mohali), Punjab 160 062, India

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ABSTRACT

The adverse alterations in mitochondrial functions can affect neuronal function negatively, as they play a crucial role in neuronal plasticity and death. Direct measurements of mitochondrial activity, including membrane potential and ATP production, are not easily achieved in post-mortem brain and liver samples because most organ functions cease to work after death; in fact, with increasing post-mortem intervals (PMI), the brain and liver tissues deteriorate rapidly. Standard procedures of mitochondria isolation, protein determination expressed as BSA equivalent, and spectrophotometric assessment of pore opening at 540 nm were employed. Our results showed that (a) intact mitochondria may be isolated from rat brain and liver of these rats after storage in animal body (in situ) at −20 °C for 7 days (168 h, post-mortem), (b) some population of these mitochondria can still take up exogenous Ca^{2+} and (c) they can still resist osmotically induced large amplitude swelling in a suitable buffer. The need for mitochondrial purity, structural integrity and abundance for functional studies are common hindrances that can encumber mitochondrial research. Therefore, this study is significant to have shown that PMI up to 7 days did not extensively, diminish MMPT pore status in normal and diabetic, ovariectomised rats. This can be relevant for forensic data mining.

1. Introduction

It is well documented that mitochondria perform a constitutional role in normal brain and liver functions and their disease processes. The adverse alterations in mitochondrial functions can negatively affect neuronal plasticity and death (Nakula et al., 2005; Todorova and Blokland, 2017). It is generally perceived that direct measurements of mitochondrial activity, such as membrane potential and ATP production, are not possible in post-mortem brain and liver samples. This is a general belief because most organelle functions cease after death; with increasing post-mortem intervals (PMI), the brain and liver tissues deteriorate rapidly. The need for mitochondrial purity, structural integrity, and abundance for functional studies are common hindrances that can encumber mitochondrial research (Barksdale et al., 2010).

The brain is an immediate target of mitochondrial aging (Aliev et al., 2009; Boveris and Navarro, 2008; Reddy and Beal, 2008), and as a great consumer of energy and oxygen, it is highly susceptible to mitochondrial decay majorly from oxidative stress, resulting from cellular Ca^{2+} fluxes as observed in aging and age-related neurodegenerative conditions including Alzheimer's and Parkinson's diseases (Nakula, 2007; Dong et al., 2011). Energy demands of the adult healthy brain are extremely high, running to 20% while itself is only about 2% of body weight (Ashmore et al., 1972; Kuzawa and Blair, 2019).

Perturbations in the communication pathways between the liver and the brain can give rise to changes in central neural activity. The mechanisms governing these changes within the brain are not fully understood to the same extent as the already fact that obesity-associated insulin resistance affects the liver and adipose tissue (Mighiu et al., 2012). The proposition of whether obesity induces inflammation in the brain in such a manner that can disrupt the control of glucose

* Correspondence to: Biochemistry Department, Faculty of Basic and Applied Sciences, College of Science, Engineering and Technology, Osun State University, Osogbo, P.M.B. 4494, Osogbo, Nigeria.
E-mail address: ebenezer.ajayi@uniosun.edu.ng (E.I.O. Ajayi).

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homeostasis by insulin was only recently elucidated using rodents (Wang et al., 2008). Therefore, the liver-brain inflammation axis has been hypothesized to be a plausible target for novel therapeutics that can reduce the burden of symptoms in patients with liver disease (D'Mello and Swain, 2011).

Decay is evident in the brain tissue as a selective loss of brain cells in areas associated with mobility, learning, and memory, which is why those functions deteriorate most rapidly with aging (Manson et al., 2006). The concept of neuroprotective effects of estrogen in women remains controversial because these effects may vary with the timing of treatment (Siegfried, 2007; Shankar, 2007). Estrogens may have either protective or damaging effects on the brain depending on age, type of menopause (natural versus surgical), or stage of menopause (Walter et al., 2010). In the mitochondria, estrogen receptors play a pivotal role in regulating energy expenditures, and protection from oxidative stress (Brinton, 2008; Simpkins et al., 2009). Mitochondrial actions provide a model for assessing the relations among menopause, estrogen exposures, and Alzheimer's disease (Henderson and Brinton, 2010) and estrogen effects on mitochondrial function seem particularly germane to this reduction (Kroemer and Reed, 2000).

Programmed cell death, or apoptosis, is activated by a broad range of signals or cascade of events and can be initiated via an intrinsic pathway involving release of cytochrome c and other proteins from the mitochondrial membrane space (Kroemer and Reed, 2000). The mitochondrial membrane permeability transition pore is a simple assessment for programmed cell death, and is suitable to measure apoptotic cell death under post-mortem conditions. The distribution and characteristic behaviour of the pore is an early appearance of the transition upon exogenous Ca2 + accumulation, and mitochondrial swelling possible culminating in programmed cell death once apoptotic factora are released.

Estradiol increases the expression of B-cell lymphoma (Bcl) anti-apoptotic proteins Bcl-2 and Bcl-XL (Garcia-Segura et al., 1998; Pike, 1999) located mainly in the outer membrane, rendering neurons less vulnerable to apoptosis. Calcium sequestration within mitochondria in response to estradiol reduces neuronal vulnerability to glutamate excitotoxicity (Brewer et al., 2006) another potential trigger for apoptosis. Estrogen-containing hormone therapy improves brain bioenergetics among older post-menopausal women, who, in most instances have presumably used hormone therapy over a long period of time (Schönknecht et al., 2003; Nilsen et al., 2006). In this study, we sought to investigate the effect of PMI on MMPT pore status of control, and insulin-resistant, ovariectomised rats; if (a) intact mitochondria can be isolated from rat brain and liver of these rats after storage in animal body (in situ) at −20 °C for 7 days (168 h, post-mortem), (b) these mitochondria can still take up exogenous Ca^{2+} and (c) they can still resist osmotically induced swelling in Mannitol: Sucrose: HEPES (MSH) buffer.

2. Materials and methods

2.1. Chemicals

Mannitol, sucrose, 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), sodium succinate, and other chemicals were products of Sigma Chemical Co. (St. Louis, MO USA). Except otherwise stated, all other solvents used were of analytical grade.

2.2. Animal care

Twenty-five female Sprague-Dawley rats (8−9 weeks old) weighing 200 ± 10 g were used for the experiment. All animals were maintained under standard conditions and control groups were rodent normal pellet diet ad libitum, while test groups manipulated for T2DM were fed on high fat diet. The animals were allowed access to water during fast for 12 h before sacrifice by decapitation. Experiments were conducted using five animals per group. The animals were handled under humane conditions in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, as well as with ethical approval granted by the Institutional Animal Care and Use Committee of the National Institute of Pharmaceutical Education and Research, S. A. S. Nagar, Punjab, India (NIPER-S, IAEC/10/54).

2.3. Induction of non-obese type 2 diabetes mellitus

The induction of non- obese diabetes was done following the method described be Srinivasan et al. (2005), high fat diet [(HFD) 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal] was given to animal groups 2–5 CE libitum, for the initial period of 2 weeks, and continuously throughout the treatment period that lasted 8 weeks (1 week during which low-dose STZ was given *i.p.*— 35 mg/kg; and 21 days of extract intubation).

2.4. Surgical procedure for ovariectomy

Using ketamine (80 mg/kg)/xylazine (8 mg/kg) anaesthesia (administered *i.p.*), the female rats were ovariectomised after being fed a high-fat diet for 10 weeks in order to mimic post-menopausal metabolic syndrome (PMS) in older women according to the method earlier reported by Mittal et al. (2009) and Bendale et al. (2013).

2.5. Experimental design

Ovariectomy was performed after diet manipulation of animals according to the grouping. Only rats with plasma glucose levels ≥250 mg/dl were selected for the experiment. The method of the sacrifice of these rats by decapitation may cause the brain and liver to undergo a little degree of ischemic-reperfusion. Following decapitation, the carcass was stored at −20 °C for 7 days to simulate post-mortem conditions. They were then thawed, and the livers and brains were harvested for mitochondria isolation.

2.5.1. Animal grouping

The experimental animals were randomly divided into 5 groups: (1) a control non-ovariectomised group fed normal pellet diet; (2) a control non-ovariectomised group fed high -fat diet; (3) an ovariectomised group fed normal pellet diet; (4) an ovariectomised group fed high-fat diet; (5) an ovariectomised, β-estradiol-treated group fed high-fat diet. High-fat diet – HFD (58% fat, 25% protein, and 17% carbohydrate, as a percentage of total kcal) was given to animals in groups 2, 4 and 5 CE libitum throughout the study that lasted 18 weeks. This serves as a model for obesity and insulin resistance (Srinivasan et al., 2005).

2.6. Isolation of rat liver mitochondria

Rat liver was weighed and washed in isolation buffer (Buffer C) containing 210 mM mannitol, 70 mM sucrose and 1 mM EGTA in 5 mM HEPES-KOH, pH 7.4. A 50% suspension was prepared by homogenising the liver in a glass-Teflon homogeniser. The homogenate was centrifuged at 2300g for 5 min twice in a SIGMA-6K15 refrigerated centrifuge to sediment the nuclear fraction and cell debris, and to remove unbroken cells by low speed centrifugation. The supernatant was centrifuged at 13000g for 10 min to pellet the mitochondria. The brown mitochondria pellet was washed twice by re-suspending in isolation buffer containing 0.5% BSA and centrifuged at 12000g for 10 min. Mitochondria were suspended in buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4 and 0.5 mM KH2PO4 and dispensed in Eppendorf tubes kept on ice (Diane and Henry, 1967; Hogeboom et al., 1948; Lapidus and Sokolove, 1994; Schneider and Hogeboom, 1948).

2.7. Isolation of rat brain mitochondria

Whole brain was excised, minced and homogenised Buffer C (1:5, w/v) using glass-Teflon homogeniser. The homogenate was ccentrifuged at 600g for 8 min at 4 °C. Brain mitochondria were obtained by centrifugation of supernatant at 5500 x g for 15 min. Pellets were washed in isolation buffer containing 0.5% BSA at 5500g for 15 min. Pellets were suspended in 500 µl of isolation buffer without BSA. Mitochondrial fractions were analysed within 4 h of isolation (Hogeboom et al., 1948; Barksdale et al., 2010).

2.8. Protein content determination

Protein content was determined using the method of Lowry et al. (1951) using bovine serum albumins (BSA) as standard.

2.9. Treatment of mitochondria

Brain and liver mitochondrial proteins (80–100 mg/ml and 1 mg/ml, respectively) were incubated with the assay buffer containing 210 mM mannitol, 70 mM sucrose in 5 mM HEPES-KOH, pH 7.4. The incubation of mitochondria was done with 20 µM rotenone for 3 min prior to the addition of 12 mM CaCl2, thirty seconds after which the assay was energised by 50 mM sodium succinate. Mitochondria swelling was spectrophotometrically measured by continuous time scan of the change in absorbance at 540 nm for 12 min (Johnson and Lardy, 1967). (Figs. 1 and 2).

2.10. Statistical analysis

Statistical analyses were performed using SPSS 18 for windows. The comparisons between groups (recorded in the absence (NTA) and presence (TA) of the triggering agent, Ca^{2+}, using the Mean ± Standard Deviation) were performed using one-way ANOVA followed by a Turkey post hoc test. $p < 0.05$ was considered statistically significant for comparison between measurements. The graphs were plotted using the values of the differences between each absorbance reading and the one read at T = 0 s, over the 12 min, calculated as:

Change in Absorbance = [Abs at T_t – Abs at T_0]·

3. Results

The degree of Ca^{2+} uptake (percentage induction of the pore opening) exhibited by the mitochondria significantly varied across the groups for both the brain (Figs. 4–8) and liver tissues (Figs. 9–13) as shown in Fig. 3. The liver showed post-mortem deterioration in the normal, non-ovariectomised group (Fig. 9). Observation for the other groups showed that 168-hour post-mortem liver mitochondria were still able to take up Ca^{2+}in a consistent time-dependent manner, though the degree of intactness in the absence of exogenous Ca^{2+} were low (Figs. 10–12).

To fully comprehend the function of mitochondria in the central nervous system, direct estimation of mitochondrial activity is typically necessary. However, one of the main hindrances to assessing mitochondrial function is that many functional assays require abundant amounts of intact mitochondria. Animal models are commonly used for the study of mitochondrial function and dysfunction in the central nervous system because they can be manipulated genetically and pharmacologically, and because they are an abundant source of mitochondrial preparations (Barksdale et al., 2010; Keri et al., 2010).

High-fat feeding induces stress in the hypothalamus of rodents (Ozcan et al., 2009). Obesity is not only associated with hypothalamic inflammation, it is also known to activate a brain-liver circuit which inhibits glucose production in obese rodents. Dwindling estrogen levels in menopause (Zhang et al., 2008) including pre- and post-menopausal stages also predisposes women to fat accumulation, weight gain, and mid-life or late-onset set obesity (Davis et al., 2012; Lizcano and Guillermo Guzmán, 2014). The onset of menopause in mid-life also increases the likelihood to develop Alzheimer's disease in these women. However, independent of changes in body weight and hepatic lipid accumulation, inhibition of diet-induced hypothalamic inflammation restores the ability of insulin to stimulate hepatic signal transduction and suppress

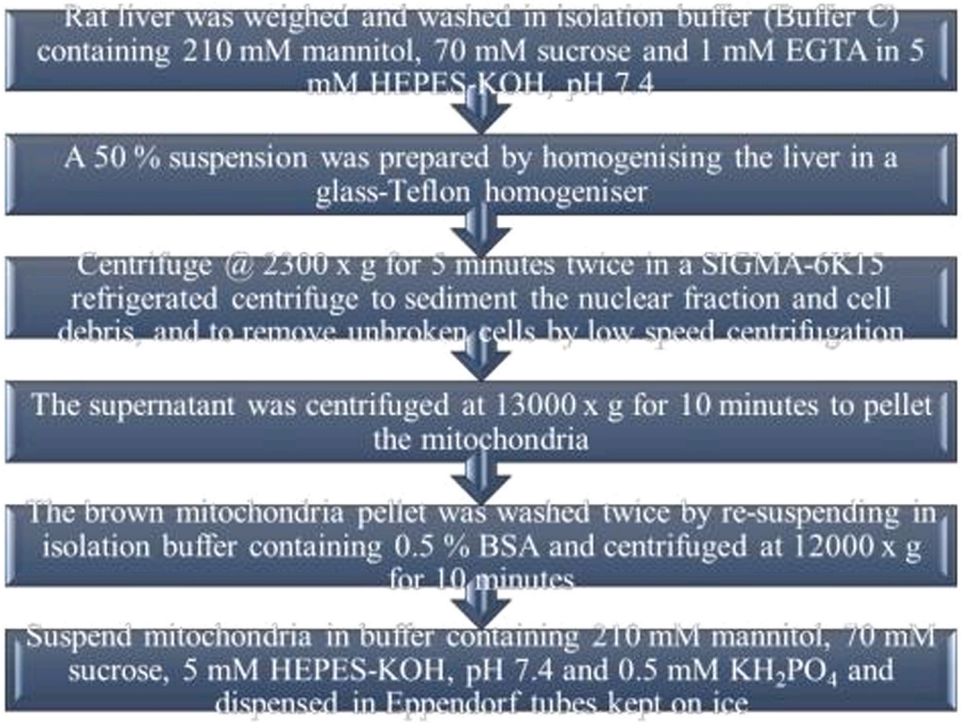

Fig. 1. Excision of organs and isolation of Isolation of rat liver mitochondria.

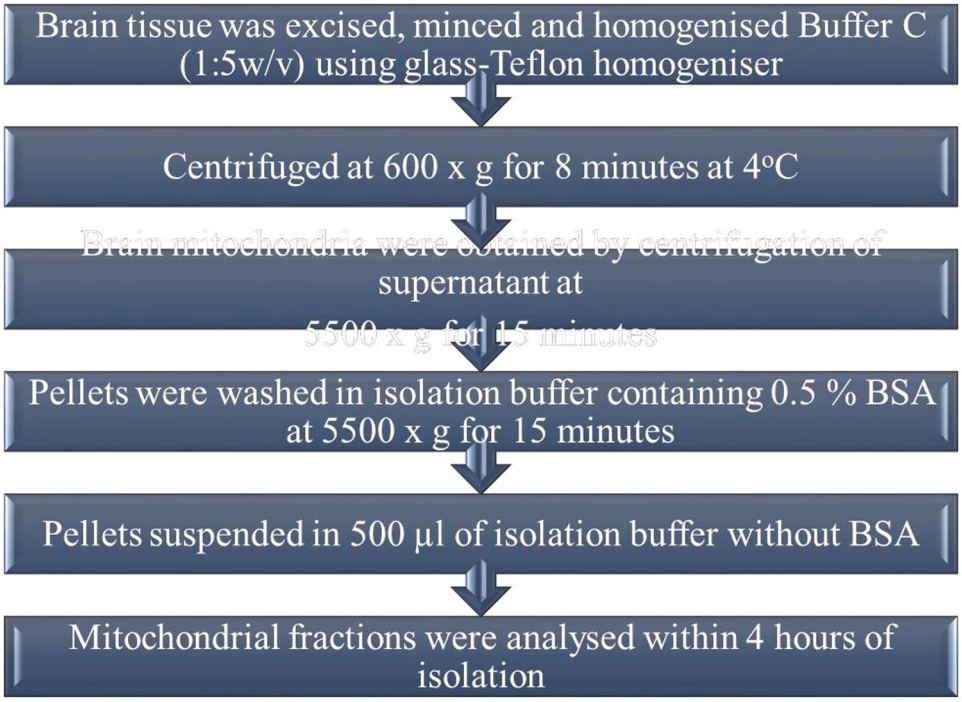

Fig. 2. Isolation of rat brain mitochondria.

glucose production in obese rodents. This shows that weight loss alone achieved by whatever method cannot enhance hepatic insulin sensitivity (Christensen and Christian, 2015).

In the brain, normal diet without menopause showed apparently intact mitochondrial ab initio before treatment with exogenous Ca^{2+}. They also showed capability to respond to Ca^{2+} uptake leading to a quite large but stable amplitude swelling over a considerable period of time (Fig. 4). However, with menopause, the mitochondria permeability transition pore appeared to be stable over a short period of time; although the integrity could not be sustained in a time-dependent fashion. The mitochondria still responded to triggering by exogenous Ca2 + , which caused a low amplitude swelling that slowly progressed in a time-dependent fashion (Fig. 6). High fat diet feeding appeared to have led to the loss of integrity in the mitochondria population, which showed inability to respond to exogenous Ca^{2+} triggering, since the pore was already opened under post-mortem condition (Fig. 5). High fat diet feeding in menopause seemed to have left the mitochondria deteriorated in the postmortem condition. Their integrity was lost, and they did not respond early to triggering by exogenous Ca^{2+} up to the ¼th of the assay time, following which there was a quick progression of low amplitude swelling (Fig. 7). Lastly, the effect of estradiol on menopause, coupled with high fat diet feeding appeared to have preserved the integrity of the mitochondrial, postmortem. Whereas the mitochondria did not respond to Ca2 + triggering, rather, the pore closure could be observed. As such, the mitochondria appeared to be protected from possibly causing programmed cell death (Fig. 8).

In the liver, the mitochondria of the apparently normal, non-menopausal rats, although responded to triggering by exogenous Ca^{2+} half-way into the measurement, the recorded values were not sufficient to indicate pore opening, compared to the observed values in the absence of exogenous Ca^{2+} (Fig. 9). The mitochondria of the normal pellet diet-fed, ovariectomised rats were intact before treatment with exogenous Ca^{2+}. Menopause seemed to have led to a fairly high amplitude swelling in response to Ca^{2+} triggering even though the animals were not exposed to high fat diet in a time-dependent manner (Fig. 11). The mitochondria of high fat diet-fed, non-menopausal rats also appeared to be intact ab initio in the absence of Ca2 + triggering. Despite the absence of menopause, high fat feeding still led to a quite high amplitude swelling of the pore in response to Ca^{2+} triggering, postmortem (Fig. 10). High fat diet and menopause seemed to have preserved the integrity of mitochondria, postmenopausal, in the absence of Ca^{2+}. Upon triggering with exogenous Ca^{2+}, the mitochondria displayed a slowly progressing, fairly large amplitude swelling. Menopause appeared to be a disadvantage, especially with high fat feeding (Fig. 12). Lastly, attempted reversal of menopause by β-estradiol appeared to have caused a transiently progressing low amplitude swelling despite high fat feeding. The slow progression of the pore opening appeared not to be smooth, possibly depicting a transient spike of cytosolic wave.

4. Discussion

Interestingly, estrogen-inducible neuroprotective mechanisms converge into mitochondria, which are pivotal to sustaining calcium homeostasis and cell survival (Morrison et al., 2006). Estrogen-activated cellular signaling cascade promotes enhanced mitochondrial function, leading to increased calcium load tolerance, enhanced electron transport chain efficiency, and promotion of antioxidant defense mechanisms (Eberling et al., 2004; Simpkins et al., 2005). These actions are mediated by the regulation of both nuclear and mitochondrial encoded genes initiated by the activation of second-messenger signaling cascades. Simultaneous activation of two pathways that prevent mitochondria from activating cell-death cascades is likely to promote neuron survival (Morrison et al., 2006). Mitochondrial dysfunction disrupts calcium homeostasis and increases oxidative stress within apoptotic subpopulations of neurons, as proposed to underlie neurodegenerative pathologies and associated cognitive decline (Simpkins et al., 2005). Bcl-2 expression potentiates the maximal intra mitochondrial calcium uptake capacity (Murphy et al., 1996). To prevent apoptosis, mitochondrial calcium load tolerance is increased by estrogen in primary cultured hippocampal neurones and isolated rat brain mitochondria (Simpkins et al., 2005).

A phyto-oestrogen combination was designed to optimally target ER β in the brain (Zhao et al., 2009; Henderson and Brinton, 2010) for neuroprotective effect with bioenergetic efficacy in the ovariectomised (OVX) rat model and long-term efficacy in a preclinical mouse model of human menopause. Recombinant ER α and ER β can directly bind

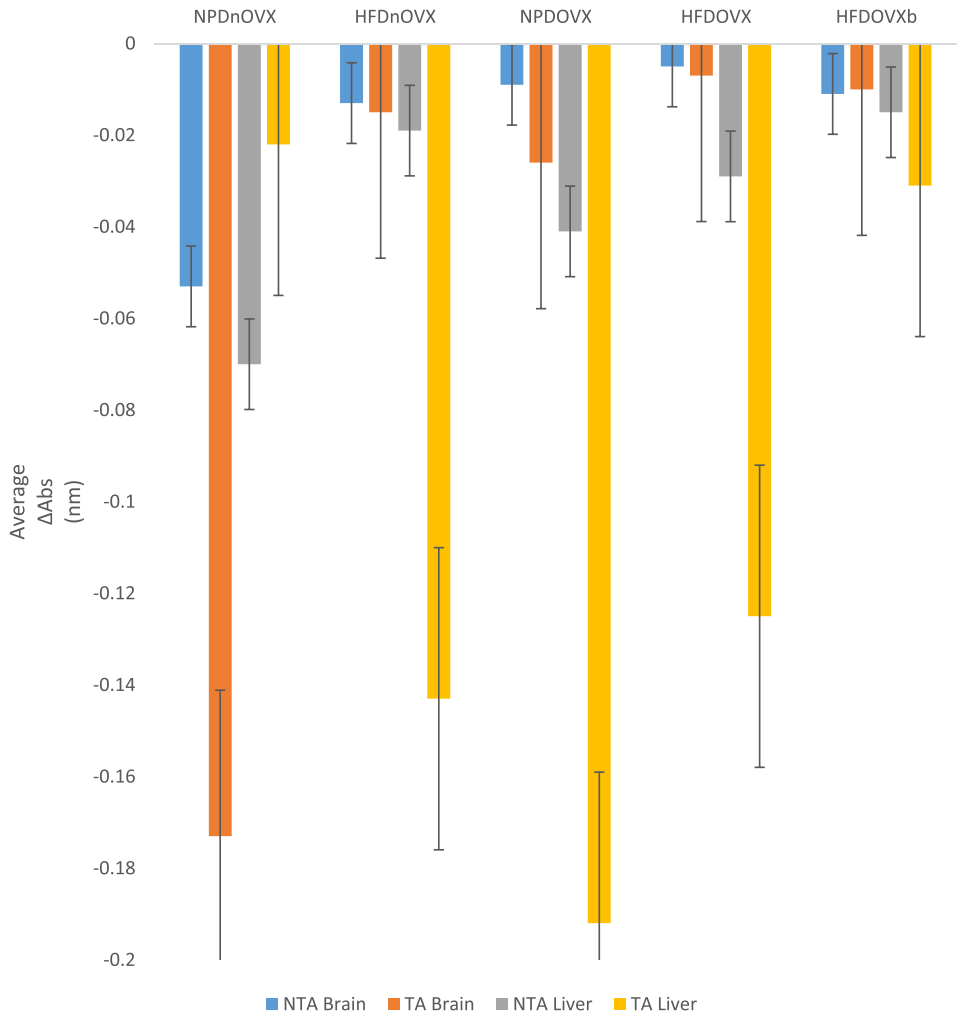

Fig. 3. Comparison of the average changes in absorbance of postmortem brain and liver mitochondria of insulin-resistant, ovariectomised rats measured in vitro at 168 h, post-mortem.

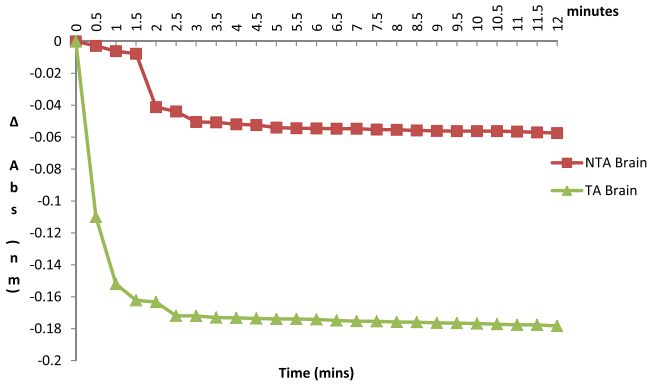

Fig. 4. Post-mortem MMPT pore opening in brain of normal diet non-ovariectomised rat.

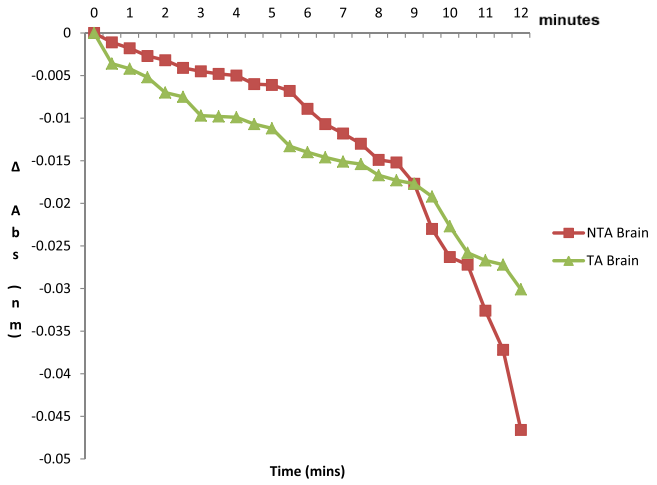

Fig. 5. Post-mortem MMPT pore opening in brain of high fat diet non-ovariectomised rat.

mitochondrial (mt) DNA through interactions with mitochondrial oestrogen response elements and that the binding was increased by E2 (Garcia-Segura et al., 1998; Pike, 1999). Estradiol increased mitochondrial sequestration of Ca^{2+} and protected neurons against adverse consequences of excess cytoplasmic Ca^{2+} and subsequent dysfunctions in the regulation of Ca^{2+} homeostasis. Despite an increased mitochondrial Ca^{2+} load, estradiol preserved mitochondrial respiratory capacity (Murphy1996) Subsequent analyses demonstrated that estradiol regulated calcium homeostasis in healthy neurons derived from middle-aged and aged rat hippocampus as well as those derived from embryonic rat hippocampus (Chen et al., 2006). However, increasing Ca^{2+} influx and Ca^{2+} sequestration into cytoplasmic and mitochondrial

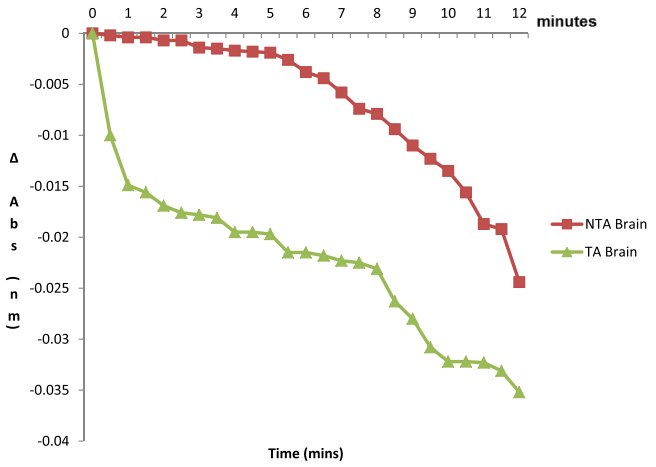

Fig. 6. Post-mortem MMPT pore opening in brain of normal diet ovariectomised rat.

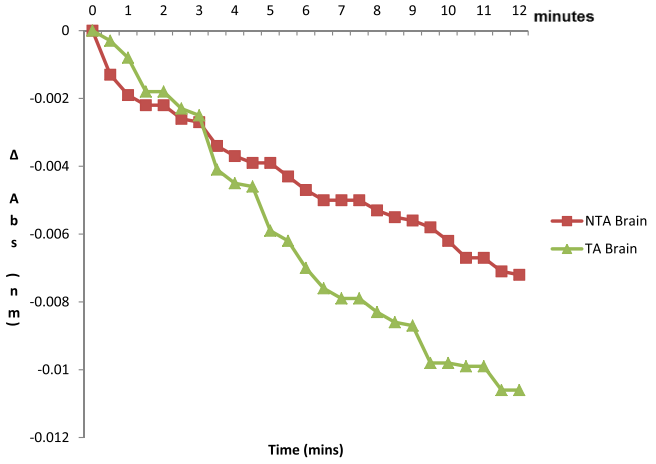

Fig. 7. Post-mortem MMPT pore opening in brain of high fat diet ovariectomised rat.

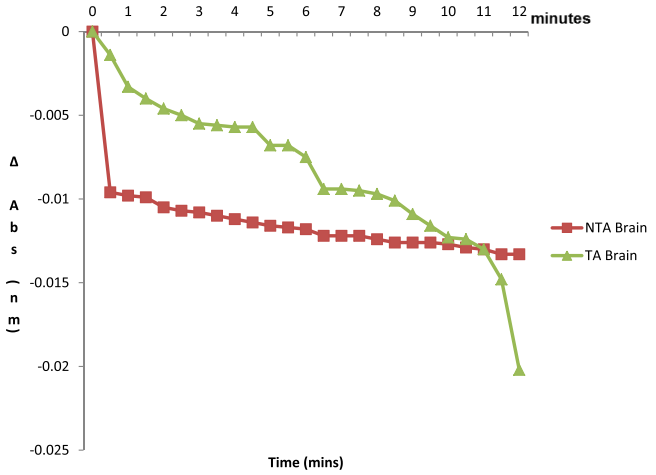

Fig. 8. Post-mortem MMPT pore opening in brain of high fat diet ovariectomised estradiol (i.p) rat.

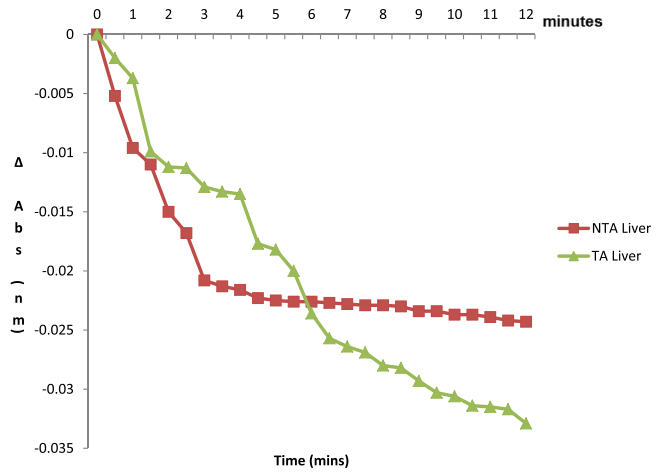

Fig. 9. Post-mortem MMPT pore opening in liver of normal diet non-ovariectomised rat.

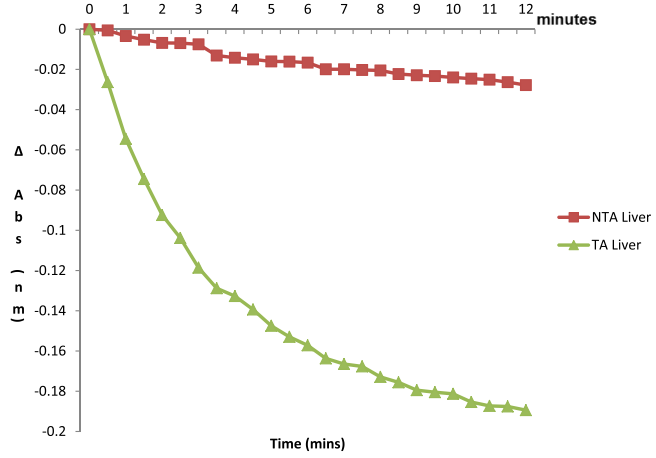

Fig. 10. Post-mortem MMPT pore opening in liver of high fat diet non-ovariectomised rat.

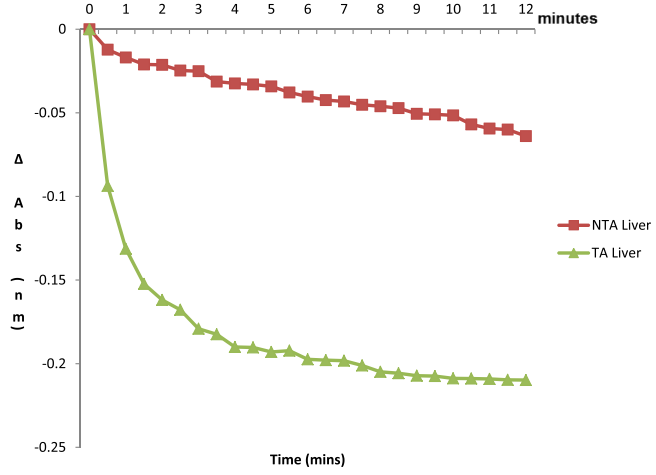

Fig. 11. Post-mortem MMPT pore opening in liver of normal diet ovariectomised rat.

compartments of neurons unable to maintain Ca^{2+} homeostasis leads to exacerbation of Ca^{2+}-dependent degenerative insults (Zhao et al., 2011).

Postmortem organ donation after a circulatory arrest (brain death) are forbidden in many clinical instances, while those that have in situ ventricular drain (for CSF drainage), are sometimes or always removed if a decision to withdraw life-sustaining measures is reached in a patient

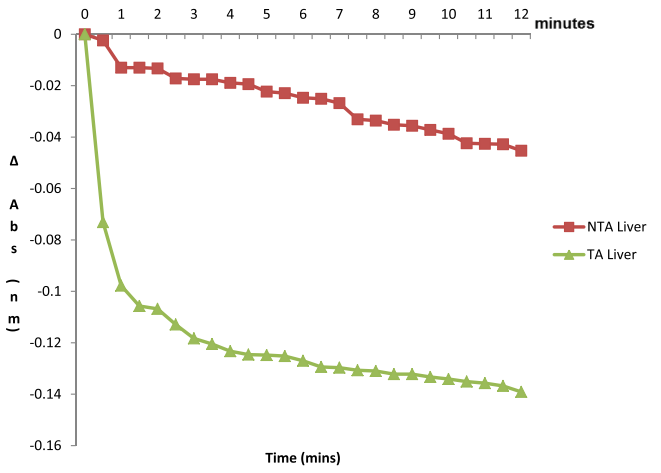

Fig. 12. Post-mortem MMPT pore opening in liver of high fat diet ovariectomised rat.

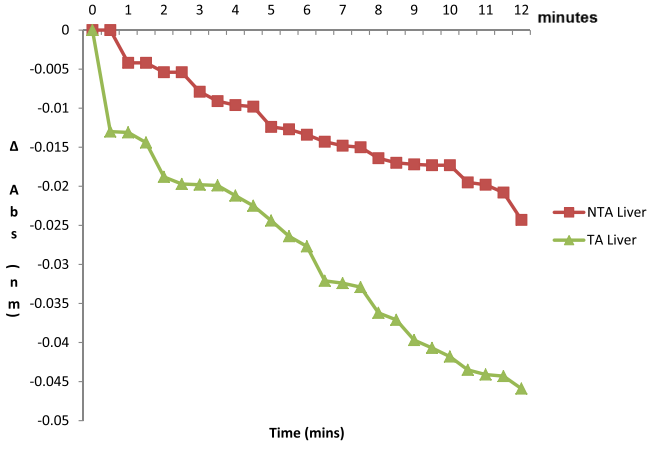

Fig. 13. Post-mortem MMPT pore opening in liver of high fat diet ovariectomised estradiol (i.p) rat, NTA (No Triggering Agent): Measurements in the absence of exogenous Ca^{2+}, TA (Triggering Agent): Measurements in the presence of exogenous Ca^{2+}.

with high intracranial pressure, but who is not brain dead. As such, we directly measured post-mortem mitochondrial swelling in normal and diabetic, ovariectomized rats because tissues deteriorate rapidly and their functions cease to work after death; especially with increasing post-mortem intervals (PMI).

Postmortem donor liver transplantation is very important and common in many parts of the world, where the availability of postmortem donor organs has become insufficient to take care of the increasing demand for transplantable organs. As a consequence, waiting lists for most types of organ transplantation are annually growing rapidly, whereas donor livers may even become unsuitable for transplantation following long post-mortem intervals. This further shrinks the donor pool size at the point of organ procurement.

The brain is an important organ useful in transplantation or donation for research. Some subjects (donors) may be ill prior to their demise. Without doubt, for brain banking, a lot take place after a decision to become a future donor is made (the front-end) and consent obtained ahead of the post-mortem processing (the large middle-ground) between the decision and the brain tissue processing that takes place after the individual has died (back-end) processes.

5. Conclusion

Mitochondria perform a constitutional role in normal brain and liver functions, and in their disease states. Using ovariectomized middle-aged rats to mimic the post-menopausal pathophysiological changes in women, we have, by these functional assay demonstrated that in situ post-mortem brain and liver of high fat fed, menopausal female rats may yield mitochondria that cannot resist assault from spikes of cytosolic waves of Ca^{2+} at a PMI of 168 h. The lag phase time observed in mitochondrial swelling induced by 12 mM Ca^{2+} represents the time for intra mitochondrial Ca^{2+} accumulation which is required to open MMPT pore. The brain does not deteriorate as fast as the liver post-mortem. But observation showed that high fat diet (lifestyle), ovariectomy (menopause), and a combination of both tend to differentially enhance MMPT pore opening in 168-hour post-mortem brain, mostly showing deterioration in the quality of the organ. The other groups showed that mitochondrial pore opening was time-dependent and consistent up to 5 min only, beyond which the amplitude swelling is not consistent. The liver showed post-mortem deterioration in the normal, non-ovariectomised group. Observation for the other groups show that 168-hour post-mortem liver mitochondria were still able to take up Ca^{2+} in a consistent time-dependent manner, though the degree of intactness in the absence of exogenous Ca^{2+} were low (Figs. 10–12). β-estradiol-treated group did not open as much as those ovariectomised groups, meaning that β-estradiol may be able to restore the liver mitochondria to the pre-menopausal status (Fig. 13). Enhanced pore opening following high fat feeding indicated that bioenergetically viable mitochondria may not be abundant from those groups. Attempted menopause reversal by β-estradiol seemed to moderately abrogate the low amplitude swelling compared to the other groups in the absence of exogenous Ca^{2+}, however, not so much so in the presence of Ca^{2+}.

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Ethical statement

The animals were handled under humane with in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, as well as with ethical approval granted by the Institutional Animal Care and Use Committee of the National Institute of Pharmaceutical Education and Research, S. A. S. Nagar, Punjab, India (NIPER-S, IAEC/10/54).

CRediT authorship contribution statement

AEIO conceived the study, and participated in its design and execution and helped to draft the manuscript; ORM participated in the design, coordination and helped to draft the manuscript; AEI participated in the preparation of the manuscript; DSPT participated in the execution of the experiments; OIO participated in the coordination of the study; KBT participated in the design, coordination and overall supervision of the study. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

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