OBESITY ASSOCIATED MITOCHONDRIAL DYSFUNCTION:
EVIDENCES FROM WNIN MUTANT RATS

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ABSTRACT

WNIN/Obese mutant rats portray features of obesity such as higher body weight with hyperinsulinemia which is similar to the preclinical/clinical features of obesity induced type 2 diabetes in humans. Using this mutant model several previous studies have unequivocally demonstrated metabolic insults and suggested its application as a potential model to study the metabolic syndrome vis-a-vis elite complications. The aim of the current study was to compare the mitochondrial swelling in female WNIN/Obese (O) rats at different age (i.e., 35, 90, 180 and 360 days old) to that of WNIN-parental (W) and WNIN/Ob lean (L) rats as controls along with some physical and biochemical parameters. The whole body dual energy X-ray absorptiometry (DXA) and biochemical analysis of fasting plasma were performed. Liver mitochondria were isolated and subjected to Ca²⁺-induced swelling study. Obese rats of the designated WNIN/Obese (O) group showed decreased susceptibility to Ca²⁺-
induced swelling. Additionally, its drastically worsened physical and biochemical indices compared to WNIN-parental (W) and WNIN/Ob lean (L) suggest that obesity is one of the factors to alter the permeability of inner mitochondrial membrane at least in this experimental condition.

**KEYWORDS:** WNIN obese mutant rat; obesity; liver mitochondria; swelling; DXA.

**List of abbreviations: (alphabetical)**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ala</td>
<td>Alamethicin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>Calcium chloride</td>
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<tr>
<td>CSA</td>
<td>Cyclosporin A</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>CyP-D</td>
<td>Cyclophilin – D</td>
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<td>Cyt C</td>
<td>Cytochrome C</td>
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<tr>
<td>DXA</td>
<td>Dual energy X-ray absorptiometry</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra-acetic acid</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
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<td>L</td>
<td>Lean strain of WNIN/Obese mutant rat</td>
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<tr>
<td>LBM</td>
<td>Lean body mass</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
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<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
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<tr>
<td>MSM</td>
<td>Mannitol-sucrose-MOPS</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>O</td>
<td>WNIN/Obese mutant rat strain</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
<tr>
<td>W</td>
<td>WNIN-parental strain of WNIN/Obese mutant rat</td>
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<tr>
<td>WB</td>
<td>Whole body</td>
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<tr>
<td>WNIN</td>
<td>Wistar rat stock at National Institute of Nutrition, Indian Council of Medical Research (ICMR), Hyderabad, India.</td>
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<tr>
<td>$\Delta \psi$</td>
<td>Membrane potential</td>
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**INTRODUCTION**

The prevalence of obesity is rising at an alarming rate and becoming a major public health concern with immeasurable social costs.$^{[1]}$ It is now considered as a complex and acutely devastating nutrition-related disorder, associated with risk of non-communicable chronic diseases such as diabetes,$^{[2]}$ hypertension,$^{[3]}$ and cardiovascular diseases$^{[4]}$ along with other chronic diseases such as osteoarthritis,$^{[5]}$ cancers,$^{[6]}$ liver disorders$^{[7]}$ etc. Liver being a vital organ in maintaining the overall metabolic homeostasis, any alteration in hepatic function has
Liver diseases are associated with the accumulation of hepatic lipids which are also closely associated with metabolic syndrome, oxidative tissue damage and mitochondrial dysfunction. Mitochondria are dynamic intracellular organelles, which play central roles in energy metabolism (i.e., production of ATP and heat), generation of reactive oxygen species (ROS) and apoptosis regulation in response to cellular metabolic needs. Because of the critical roles in these important cellular functions, mitochondrial conditions are associated with a wide variety of human diseases such as neurodegenerative diseases, infertility, human malignancies, obesity and type 2 diabetes when mitochondrial membrane permeability is considered as one of the major factors.

The stimulation of the mitochondrial permeability transition pore (MPTP) can compromise mitochondrial dysfunction followed by cellular death in both apoptotic and necrotic injuries. The MPTP occurs during the initiation of permeability pathways in the inner mitochondrial membrane in response to Ca\(^{2+}\) flux and oxidative stress leading to loss of mitochondrial membrane potential (Δψ) and swelling. Stimulation of the MPTP may result in ‘cytochrome C’ release with subsequent apoptosis and loss of ATP production leading to necrosis. In stressed environment, opening of a low permeability pathway leads to mitochondrial depolarization without prominent swelling and Ca\(^{2+}\) uptake.

With reference to the above facts we hypothesized that vulnerability of liver mitochondria may be reduced during obesity due to the induction of the permeability pathways by calcium. Therefore, we compared the swelling of purified liver mitochondria, isolated from female obese mutant rats to that of controls. Since 1920, National Institute of Nutrition (NIN), Hyderabad, India has been maintaining inbred stock of Wistar rats, known as WNIN. From this stock, two more phenotypes were isolated and established as lean (WNIN/Ob lean, L) and mutant obese (WNIN/Obese, O) strains, in 1994 and are being maintained at NIN ever since. The original Wistar stock is known as parental (WNIN-parental) strain. Due to the availability of this model at our institute, the aim of our current study was to compare the mitochondrial swelling along with physical and biochemical parameters such as body weight, organ (liver) weight, fat content, lean body mass (LBM), bone mineral density (BMD) and plasma levels of fasting glucose and lipids in female WNIN/Obese (O) rats at different time points (i.e., 35, 90, 180 and 360 days) to that of WNIN-parental (W) and WNIN/Ob lean (L) rats as control. The reduction in susceptibility of liver mitochondria to Ca\(^{2+}\)-induced MPTP in
WNIN/Obese (O) mutant rats compared to controls revealed a novel link between MPTP and metabolic homeostasis of obesity.

MATERIALS AND METHODS

Reagents

All chemicals were (BioXtra and BioUltra, ≥ 98-99%) molecular biology grade and were procured from Sigma-Aldrich, U.S.A., unless otherwise indicated.

Animals

Female rats of 35, 90, 180 and 360 days old of WNIN- parental (W), WNIN/Ob lean (L) and WNIN/obese (O) phenotypes were obtained from the stock colony of National Centre for Laboratory Animal Sciences (NCLAS) at National Institute of Nutrition (NIN), Indian Council of Medical Research (ICMR), Hyderabad, India and study was approved by the Institutional Animal Ethical Committee, IAEC (P2/IAEC/NIN/2012/4/NVG). Animals were fed on standard pellet diet ad libitum with free access to drinking water for 35, 90, 180 and 360 days. At the end of each experimental period, blood was collected from retro-orbital sinus of overnight fasted animals and they were sacrificed by cervical dislocation to perform the following experiments.[22]

Dual energy X-ray absorptiometry (DXA) Scan

Fat content, lean body mass (LBM) and bone mineral density (BMD) measurements were carried out by a dual energy X-ray absorptiometry (DXA) using Hologic Discovery QDR series X-ray machine (Hologic Inc., Massachusetts, USA) of the whole body (WB), by a trained person who carried out all the scans and analyzed the results according to the manufacturer’s instructions.[23]

Biochemical parameters

Levels of glucose and lipid profile were measured in the plasma of overnight fasted animals using commercially available kits from Biosystems (Barcelona, Spain).

Mitochondrial isolation

After sacrificing the animals liver was immediately dissected out, rinsed in ice-cold mannitol-sucrose- 3-(N-morpholino) propanesulfonic acid (MOPS), MSM (0.23 M mannitol, 0.07 M sucrose, 17 mM MOPS-NaOH, pH 7.4) and chopped into small pieces. These chopped pieces were further minced and homogenized in MSM containing 0.5 mM ethylene glycol.
tetraacetic acid (EGTA) and 0.5% bovine serum albumin (BSA) at a ratio of 9 ml of homogenization medium per gram of tissue in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 g for 10 min and the collected supernatant at 8,000 g for 10 min to precipitate mitochondria that were washed twice in MSM at 10,000 g under the same condition.\textsuperscript{[24, 25]}

The pellet was resuspended in MSM and mitochondrial protein was estimated using Bradford’s reagent where BSA was used as a standard. Samples were solubilised in deoxycholate (Na\textsuperscript{+}), which was present at a final concentration of 1% by weight. All the process of isolation was conducted at 2-4°C. Mitochondrial suspensions, containing 1 mg protein/ml, were used immediately after isolation for swelling studies \textsuperscript{[25]}

**Swelling experiments**

Solute permeability in liver mitochondrial membrane was observed by light scattering method using a UV-VIS (UV-2450) spectrophotometer (Shimadzu Corporation, Kyoto, Japan) in split beam mode at A540 at 1 mg protein/ml in MSM. Each reaction of mitochondria were incubated at 37°C for 20 minutes with three different solutes such as 2 mM inorganic phosphate (Pi) followed by 60 nmol/mg protein of calcium chloride (CaCl\textsubscript{2}), 2 µM of alamethicin (Ala) and 0.5 µM of Cyclosporin A (CsA) in a quartz cuvette to allow the constant monitoring of mitochondrial swelling as apparent change by measuring absorbance at 540 nm.\textsuperscript{[25]}

A CPS-controller (Shimadzu) was attached to spectrophotometer for the temperature regulation.

**Statistical analysis**

Statistical analyses were performed using a software package, SPSS (version 19, IBM Corp., NY, USA). Homogeneity of data was examined through Levene statistics. In case of normally distributed data for more than two groups, one way ANOVA was used to test the difference of mean values among different parameters otherwise, non-parametric statistics like Khuskal Wallis and Mann Whitney tests were used. A p value of < 0.05 was considered statistically significant.

**RESULTS**

Comparisons were made among the animals of three phenotypes such as WNIN-parental (W), WNIN/Ob lean (L) and mutant WNIN/Obese (O) at different ages (i.e., at 35, 90, 180 and 360 days old) for body weight, liver weight, body compositions and swelling in the isolated mitochondria from their liver (Table 1 and Fig. 1-5).
Body and liver weight analysis
The body weight was increased more in the WNIN/Obese (O) mutant rats compared to their counterpart controls, WNIN/Ob lean (L) (52.7%) and WNIN-parental (W) (62.4%). By the age of 90 days, the increments of body weight of WNIN/Obese (O) mutant rats were 1.4 and 1.66 fold compared to the WNIN/Ob lean (L) and WNIN-parental (W) rats, respectively (Fig-1A). The liver weight of WNIN/Obese (O) mutant rats was also significantly higher (1.3 fold) as compared to both WNIN/Ob lean (L) and WNIN-parental (W) rats (Fig-1B).

Body compositions
The DXA scan analysis showed that there was a significant ($p < 0.05$) difference in fat content in all the three phenotypes of WNIN rats. The WNIN/Obese (O) mutant rats showed significantly and drastically higher body fat (Fig. 2A) as compared to WNIN-parental (W) and WNIN/Ob lean (L) rats with higher fat content in the WNIN-parental (W) than the WNIN/Ob lean (L) rats (i.e., O>W>L). But it was observed that the lean body mass (LBM) and bone mineral density (BMD) were higher in the WNIN/Ob lean (L) rats as compared to other two phenotypes with higher LBM and BMD in the WNIN-parental (W) rats (Fig.2B and C) than the mutant WNIN/Obese (O) rats (i.e., L>W>O).

Plasma glucose and lipid profile
With the progression of age, fasting glucose, triglycerides, cholesterol and HDL-cholesterol levels in plasma were increased in all three phenotypes of rats (Fig. 3A-D). The WNIN/Obese (O) mutant rats showed significantly higher levels of fasting glucose, triglycerides, cholesterol and HDL-cholesterol ($p < 0.05$) as compared to that of WNIN-parental (W) and WNIN/Ob lean (L) rats (Fig. 3A-D) when, WNIN/Ob lean (L) rats showed higher fasting plasma glucose and triglycerides levels than that of WNIN-parental (W) rats (i.e., O>L>W) (Fig. 3A, B). On the other hand, WNIN-parental (W) rats showed higher levels of plasma lipids than the WNIN/Ob lean (L) rats (i.e., O>W>L) (Fig. 3C, D).

Mitochondrial swelling
Mitochondria were isolated from liver of WNIN rats and incubated in MSM medium to appropriate cytosolic condition. Then the addition of Ca$^{2+}$ characteristically created a momentary depolarization as its electrophoretic entry and successive damages by respiratory fluxes, afterwards a steady-state depolarization specified the level of mitochondrial permeability transition pore (MPTP).[20]
The mitochondria from younger rats were more sensitive than the older rats to Ca\(^{2+}\) depolarizing and swelling at 60 nmol/mg protein [Ca\(^{2+}\)] (Fig.4-5). Furthermore, mitochondria from rats of different ages (i.e., 35, 90, 180 and 360 days old) showed a lower pattern of swelling to Ca\(^{2+}\) in all the three phenotypes (Table-1) with the increasing age. In reference to 1 mg of mitochondrial protein per ml of the reaction volume, the amount of swelling observed in WNIN-parental (W) rats were 38.56\% at 35, 36.26\% at 90, 34.89\% at 180 to 32.54\% at 360 days of age; in WNIN/Ob lean (L); 45.37\% at 35, 44.51\% at 90, 43.13\% at 180 and 41.93\% at 360 days of age and in WNIN/Obese (O) and 43.51\% at 35, 33.05\% at 90, 31.13\% at 180 and 28.41\% at 360 days of age. Thus, Ca\(^{2+}\) pulses (60 nmol Ca\(^{2+}\)/mg protein) caused a complete sustained depolarization of mitochondria from younger rats, but not from older rats. Since the amount of swelling in the mitochondria was varied with the age (i.e., O>W>L) and phenotypes (i.e., L>W>O, except at 35 days where it was L>O>W), alamethicin (2 µM) was used as a reference for the large-amplitude maximal swelling in this experiment. Both the sustainability of Ca\(^{2+}\)-induced depolarization and large-amplitude swelling with alamethicin are typical manifestations of the standard mitochondrial permeability transition pore (MPTP).\(^{[26]}\) Thus, the results of this study clearly indicate that Ca\(^{2+}\)-depolarized swelling varies with the age and phenotypes of rats.

Cyclosporin A (CsA), a challenger of the mitochondrial permeability transition pore (MPTP) and ligand for Cyclophilin-D, (CyP-D),\(^{[27], \ 28]}\) efficiently avert the mitochondrial depolarization and temporary swelling in response to Ca\(^{2+}\). In the present study, to rule out any presence of Ca\(^{2+}\) in the swelling reactions CsA (0.5 µM) was used even though the chemicals and reagents were extra pure. However, a negligible amount of mitochondrial recovery was observed with age (Fig. 4A, B; 5A-a,b and 5B-a, b,) in both WNIN-parental (W) and WNIN/Ob lean (L) rats, when opposite result was observed for WNIN/Obese (O) mutant rats (Fig.4C; 5A-c and 5B-c, d).

Table-1: Percentage of swelling with Ca\(^{2+}\) in the three phenotypes of WNIN rats w.r.t. to 1 mg mitochondrial protein per ml of reaction.

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>% Swelling with Ca(^{2+})</th>
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<tr>
<td></td>
<td>Age (days)</td>
</tr>
<tr>
<td></td>
<td>35</td>
</tr>
<tr>
<td>WNIN-parental (W)</td>
<td>38.56</td>
</tr>
<tr>
<td>WNIN/Ob lean (L)</td>
<td>45.37</td>
</tr>
<tr>
<td>WNIN/Ob (O)</td>
<td>43.51</td>
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Cyclophilin-D (CyP-D), a ligand for MPTP, is known to efficiently avert the mitochondrial depolarization and temporary swelling in response to Ca\(^{2+}\). In the present study, to rule out any presence of Ca\(^{2+}\) in the swelling reactions CsA (0.5 µM) was used even though the chemicals and reagents were extra pure. However, a negligible amount of mitochondrial recovery was observed with age (Fig. 4A, B; 5A-a,b and 5B-a, b,) in both WNIN-parental (W) and WNIN/Ob lean (L) rats, when opposite result was observed for WNIN/Obese (O) mutant rats (Fig.4C; 5A-c and 5B-c, d).
Fig. 1: Growth pattern of female WNIN rats: WNIN-Parantal (W), WNIN/Ob lean (L) and mutant WNIN/Obese (O). A. Body weight (g) and B. Liver weight (g). Values are ± S.E. of six rats (by two way ANOVA followed by post-hoc least significance, P<0.05). Different letters over the bars for a given age are significantly different from each other phenotype of animals.
Fig. 2: Body composition in females of all the three phenotypes of WNIN rats viz., WNIN-parental (W), WNIN/Ob lean (L) and WNIN/Obese (O). A. Fat: Fat content, B. LBM: Lean body mass, and C. BMD: Bone mineral density. Each bar represents a mean of observations in six rats. Line above each bar is SEM. Different letters over the bars for a given age are significantly different from each other phenotype of animals. One-way ANOVA followed by post-hoc least significant test.
Fig. 3: Analysis of fasting glucose (Gluc), triglycerides (TG), cholesterol (Chol) and HDL cholesterol level (mg/dl) in blood plasma of female of all the three phenotypes of WNIN rats viz., WNIN-parental (W), WNIN/Ob lean (L) and WNIN/Obese (O). Each bar represents the mean of observations in six rats. The line above each bar is SEM. Different letters over the bars for a given age are significantly different from each other phenotype of animals. Two-way ANOVA followed by post-hoc least significance test.
Fig. 4A: Swelling of mitochondria isolated from female WNIN-parental (W) rats of four different ages. a. 35 days old W rat; b. 90 days old W rats; c. 180 days old W rats and d. 360 days old W rats. Mitochondria were incubated at 37 °C for 20 minutes as described in the methods and the legends to figure W-35-mito describes only mitochondria isolated from 35 days old WNIN-parental rats with no further additions; W-35-Pi+Ca describes mitochondria isolated from 35 days old WNIN-parental rats with 2 mM inorganic phosphate (Na+) addition at 2 minutes followed by CaCl2 (60 nmol/mg protein) addition at 4 minutes; W-35-Ala describes mitochondria isolated from 35 days old WNIN-parental rats with 2 µM alamethicin addition at 2 minutes and W-35-CsA describes mitochondria isolated from 35 days old WNIN-parental rats with 0.5 μM CsA present from the beginning of the incubation. Each experiment was repeated three times and it showed the similar profile.
Fig. 4B: Swelling profiles of mitochondria isolated from female WNIN/Ob lean (L) rats of four different ages. A. 35 days old L rat; B. 90 days old L rats; C. 180 days old L rats and D. 360 days old L rats. Mitochondria were incubated at 37 °C for 20 minutes as described in the methods and the legends to figure L-35-mito describes only mitochondria isolated from 35 days old WNIN/Ob lean rats with no further additions; L-35-Pi+Ca describes mitochondria isolated from 35 days old WNIN/Ob lean rats with 2 mM inorganic phosphate (Na+) addition at 2 minutes followed by CaCl2 (60 nmol/mg protein) addition at 4 minutes; L-35-Ala describes mitochondria isolated from 35 days old WNIN/Ob lean rats with 2 µM alamethicin addition at 2 minutes and L-35-CsA describes mitochondria isolated from 35 days old WNIN/Ob lean rats with 0.5 µM CsA present from the beginning of the incubation. Each experiment was repeated three times and it showed the similar profile.
Fig. 5A: Change in absorbance during mitochondrial swelling with various incubations in females of three different phenotypes at four different ages i.e., at 35, 90, 180 and 360 days. a. WNIN-parental (W); b. WNIN/Ob lean (L) and c. WNIN/Obese (O). Legends in figure mito describes only mitochondria with no further additions; Pi+Ca describes mitochondria with 2 mM inorganic phosphate (Na+) addition at 2 minutes followed by CaCl$_2$ (60 nmol/mg protein) addition at 4 minutes; Ala describes mitochondria with 2 µM alamethicin addition at 2 minutes and CsA describes mitochondria with 0.5 µM CsA present from the beginning of the incubation. Each bar represents the mean of observations in three rats. The line above each bar is SEM. Different letters over the bars for a given age are significantly different from each other phenotype of animals. Two-way ANOVA followed by post-hoc least significance test.
DISCUSSION

Worldwide outbreak of obesity both in adults and children is evolving as one of the major health issues and is normally linked with several diseases with lofty mortality and morbidity for instance cardiovascular diseases, type 2 diabetes, cancer, arthritis, hypertension, myocardial infarction etc.[29, 30] To understand the pathophysiology of obesity and related complications rodent models have provided useful insights in the previous years.[21, 31, 32]

Keeping this fact in mind, WNIN/Obese (O) mutant rats has been established earlier at the National Institute of Nutrition (NIN), Hyderabad, India which is being used as a suitable model to study the preclinical changes in obese condition.[21] Female rats were chosen as they are the sole contributor of mitochondria to their offspring and subsequent generations.[33] Any change(s) in the efficiency of females’ mitochondria increases the chances of passing on that error(s) to their offspring.[34] Therefore, in the present work, we studied the effect of obesity on mitochondrial swelling along with other parameters such as body weight, organ weight, fat content, lean body mass (LBM), bone mineral density (BMD) and some biochemical parameters like plasma level of fasting glucose (Gluc), triglycerides (TG), total cholesterol (Chol) and HDL-cholesterol (HDL) in these female WNIN/Obese (O) mutant rats. A significant increase of both physical and biochemical parameters was observed in these female WNIN/Obese (O) mutant rats as compared to their counter parts WNIN-parental (W) and WNIN/Ob lean (L).

The above-mentioned parameters were increased due to the development of hyperphagic, polydipsic, polyuric and hyperinsulinemic conditions which are closely associated with obesity.[21] Additionally, decreasing BMD along with high fat deposition with age ultimately leads to the osteoporosis which is also one of the factors associated with obesity.[21, 35, 36] The WNIN/Obese (O) mutant rats were observed to be euglycemic along with hypertriglyceridemic and hypercholesterolemic compared to the WNIN-parental (W) and WNIN/Ob lean (L) rats.[21] Euglycemic conditions in these WNIN/Obese (O) mutant rats may be due to the occurrence of hyperinsulinemic condition.[21] The progressively increasing obesity related parameters in WNIN/Obese (O) mutant rats are associated with abnormal metabolic processes as well as higher health risks which might affect metabolic organs along their mitochondrial alterations.

Since mitochondria are the heart of all metabolic processes, any alteration(s) to their structure(s) and function(s) may alter the whole body metabolism. Keeping these points in...
mind for the first time we studied the susceptibility of liver mitochondria to Ca$^{2+}$-induced swelling in these WNIN/Obese (O) mutant rats and compared to their counter parts viz., WNIN-parental (W) and WNIN/Ob lean (L) rats. Interestingly, it was observed that in reference to 1 mg of mitochondrial protein per ml of reaction the amount of overall changes in swelling were decreased with increasing age in all the three phenotypes which were more prominent in WNIN/Obese (O) mutant rats as compared to its counter parts (i.e., O>W>L). This may be inferred as occurrence of larger population of bad/leaky mitochondria in WNIN/Obese (O) mutant rats due to the deposition of more fat with age as compared to its counter parts, WNIN-parental (W) and WNIN/Ob lean (L) rats (Fig. 2 and 3). Similar observations were found in the high-fat diet (HFD) fed ob/ob mice, an in vitro model for lipotoxicity.$^{[37]}$ Accumulation of lipids in the hepatocytes of these mice led to the lack of phosphorylation of voltage-dependent anion channel (VDAC) by glycogen synthase kinase-3 (GSK3) enzyme, which compromises the permeability of the mitochondrial outer-membrane in the hepatocytes of these mice. Further, higher invasion of water and calcium into the mitochondria sensitized the organelles to matrix swelling, depolarization and release of cytochrome C (Cyt C) without provoking cell death in hepatosteatosis.$^{[37]}$ Similarly, in another study, it was observed that in obesity, uncoupling protein-2 (UCP-2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH).$^{[38]}$

In our study, the lesser amount of swelling was observed in the WNIN/Obese (O) mutant rats as compared to its counter parts (i.e., L>W>O; except at age 35 days old, it was observed as L>O>W) which suggests the presence of already swelled mitochondria due to its leaky nature in the liver of WNIN/Obese (O) mutant rats. Additionally, the amount of CsA recovery of mitochondria was observed to be little bit higher in these WNIN/Obese (O) mutant rats as compared to their counter parts.
Fig. 4C: Swelling profiles of mitochondria isolated from female WNIN/Ob (O) rats of four different ages. a. 35 days old O rats; b. 90 days old O rats; c. 180 days old O rats and d. 360 days old O rats. Mitochondria were incubated at 37 °C for 20 minutes as described in the methods and the legends to figure. O-35-mito describes only mitochondria isolated from 35 days old WNIN/Ob rats with no further additions; O-35-Pi+Ca describes mitochondria isolated from 35 days old WNIN/Ob rats with 2 mM inorganic phosphate (Na+) addition at 2 minutes followed by CaCl₂ (60 nmol/mg protein) addition at 4 minutes; O-35-Ala describes mitochondria isolated from 35 days old WNIN/Ob rats with 2 µM alamethicin addition at 2 minutes and O-35-CsA describes mitochondria isolated from 35 days old WNIN/Ob rats with 0.5 µM CsA present from the beginning of the incubation. Each experiment was repeated three times and it showed the similar profile.
Fig. 5B: Comparative change in absorbance during mitochondrial swelling with various incubations in females of three different phenotypes viz., WNIN-parental (W), WNIN/Ob lean (L) and WNIN/Obese (O) at four different ages i.e., at 35, 90, 180 and 360 days. a. 35 days; b. 90 days; c. 180 days and d. 360 days. Legends in figure mito describes only mitochondria with no further additions; Pi+Ca describes mitochondria with 2 mM inorganic phosphate (Na+) addition at 2 minutes followed by CaCl$_2$ (60 nmol/mg protein) addition at 4 minutes; Ala describes mitochondria with 2 µM alamethicin addition at 2 minutes and CsA describes mitochondria with 0.5 µM CsA present from the beginning of the incubation. Each bar represents the mean of observations in three rats. The line above each bar is SEM. Different letters over the bars for a given age are significantly different from each other phenotype of animals. Two-way ANOVA followed by post-hoc least significance test.
Thus, it can be inferred that both MPTPs’ distributions and regulations are largely affected in obesity and became worse with increased deposition of fats with age in these obese mutant rats as compared to their counter parts. Thereby, it confirms the presence of the higher population of dysfunctional mitochondria over normal mitochondria in these obese mutant rats. Further, it was also observed that in obesity mitochondrial abnormalities triggers the pathogenesis of non-alcoholic steatohepatitis.[39-41]

Current evidence links obesity and aging to insulin resistance in skeletal muscle via correlation with mitochondrial dysfunction, aberrant lipid accumulation, and oxidative stress.[42, 43] For example, physiological studies in both humans and rodents demonstrated that acute lipid infusion or chronic consumption of high-fat diet (HFD) is sufficient to promote skeletal muscle insulin resistance concomitant with lipid accumulation in muscle and/or mitochondrial dysfunction.[44, 45] Therefore, obesity associated irregularities of MPTP in dysfunctional mitochondria may be one of the contributing factors to the obesity associated metabolic complications. Moreover, hyperphagic, polydypsic, euglycemic, hyperinsulinaemic, hypertriglyceridaemic and hypercholesterolaemic conditions overloads the cells with a lot of substrates for mitochondrial oxidation (oxidative stress) thereby increases the energy content of the cells to a hyper level. This high energy state of the cells accelerates the cascade of biochemical processes such as hormonal imbalance, hormonal resistance, over activity of premature division of cell organelles and cells which further increase the entropy of the organ as well as several metabolic disorders such as diabetes, CVD, cancers etc. Hence, further study of mitochondria may pave the way for therapeutic development of obesity at cellular and molecular level.

The mitochondrial permeability transition pore (MPTP) may be a graded response connected to unpredictable or growing levels of permeabilization.[20, 46] We hypothesize that decrease in susceptibility is connected to the leakiness of mitochondrial membrane in WNIN/Obese (O) mutant rats. Thus, the difference in the mitochondrial responses in WNIN/Obese (O) mutant rats appear to be linked to the differences in the proportions of fat deposition with age in comparison to its counter parts, viz., WNIN-parental (W) and WNIN/Ob lean (L). Thus, a clear picture emerges of mitochondria during obesity that could adversely affect the function of hepatocytes before other body cells. Obesity states may also stress mitochondrial function and so the differential expression of various proteins mediating the observed Ca2+ sensitivity.
CONCLUSION
In the present study, we observed that liver mitochondrial susceptibility to Ca\(^{2+}\)-induced swelling in WNIN/Obese (O) mutant rats were decreased with the increasing deposition of fats with age which can be attributed to the progressively bad/leaky and dysfunctional mitochondria over normal and functional mitochondria in these rats. In conclusion, during obesity, the integrity of the mitochondrial membranes get significantly affected leading to the inefficient VDAC, MPTP and other ion-exchanger channels thereby making the mitochondria bad/leaky and loss of integrity related to their membrane potentials. Nevertheless, further investigations in this rat model is needed to decipher the mitochondrial parameters in the regulation of MPTP processes which would give more information to this reduced susceptibility of liver mitochondria to Ca\(^{2+}\)-induced swelling.

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Conflict of interest
The authors declare no conflict of interest.

REFERENCES


25. Martha EG, Kimberly MB, Elliott DC, Jitendra K, Gustav G, Peiffer DR. Mitochondrial iPLA2 activity modulates the release of cytochrome C from mitochondria and influences the permeability transition JBC, 2006; 281: 6931-6939.


