Tunicamycin-induced Endoplasmic Reticulum stress mediates mitochondrial dysfunction in human adipocytes

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Abstract:

Context: Dysfunctional ER and mitochondria are known to contribute to the pathology of metabolic disease. This damage may occur, in part, as a consequence of ER-mitochondria cross-talk in conditions of nutrient excess such as obesity. To date insight into this dynamic relationship has not been characterised in adipose tissue. Therefore, this study investigated whether ER stress contributes to the development of mitochondrial inefficiency in human adipocytes from lean and obese participants.

Methods: Human differentiated adipocytes from Chub-S7 cell line and primary abdominal subcutaneous adipocytes from lean and obese participants were treated with tunicamycin to induce ER stress. Key parameters of mitochondrial function were assessed, including mitochondrial respiration, membrane potential (MMP) and dynamics.

Results: ER stress led to increased respiratory capacity in a model adipocyte system (Chub-S7 adipocytes) in a concentration and time dependent manner (24hr: 23%↑; 48hr: 68%↑, (p<0.001); 72hr: 136%↑, (p<0.001)). This corresponded with mitochondrial inefficiency and diminished MMP, highlighting the formation of dysfunctional mitochondria. Morphological analysis revealed reorganisation of mitochondrial network, specifically mitochondrial fragmentation. Furthermore, p-DRP1, a key protein in fission, significantly increased (p<0.001). Additionally, adipocytes from obese subjects displayed lower basal respiration (49%↓, p<0.01) and were unresponsive to tunicamycin in contrast to their lean counterparts, demonstrating inefficient mitochondrial oxidative capacity.

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Conclusion: These human data suggest that adipocyte mitochondrial inefficiency is driven by ER stress and exacerbated in obesity. Nutrient excess induced ER stress leads to mitochondrial dysfunction that may therefore shift lipid deposition ectopically and thus have further implications on the development of related metabolic disorders.

Keywords: Obesity, ER stress, mitochondrial dysfunction, human adipocytes

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Précis

The induction of ER stress in adipocytes results in damaged mitochondrial function, the effect of which is exacerbated by conditions of obesity in primary human abdominal subcutaneous adipocytes.

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Introduction:

In the context of obesity, the adipocyte plays an essential part in balancing metabolic homeostasis in response to surplus energy. During such a period of weight gain the adipocyte is challenged with a multitude of insults, including nutrients, inflammation, and oxidative stress leading to organelle disruption and ultimately metabolic dysfunction. Although the molecular mechanisms of such obesity-induced adipose tissue dysfunction are not fully understood, disturbances of two cellular organelles in particular, the endoplasmic reticulum (ER) and mitochondria, have been widely implicated in the physiological and molecular changes that follow nutrient overload[1–4]. The excessive protein overload in obesity is detected by sensors at the ER membrane, initiating a signalling cascade known as the unfolded protein response (UPR)[5]. Three major transducers of the UPR are: activating transcription factor 6 α (ATF6α), PKR-like ER kinase (PERK) and inositol-requiring enzyme 1 α (IRE1α) which activate transient protein attenuation and transcription of protein-folding chaperones, in a bid to restore the ER functions[6]. Prior studies in mice fed a high-fat diet have shown elevated levels of ER stress related proteins, PERK and eIF2α phosphorylation as denoted in liver extracts[5] and significant upregulation of C/EBP homology protein (CHOP) in white adipose tissue[7]. The link between obesity and chronic ER stress has also been observed through studies in human tissues[4,8,9] and interventions that restore ER health; via either weight loss or drug therapies that reduce metabolic dysfunction[5,7,10].

Improvement in metabolic function may also be mediated through mitochondria as they play a key role in fatty acid esterification, glucose oxidation and lipogenesis[11]. It is therefore not surprising that metabolic imbalance in obesity is also closely linked with compromised mitochondrial function, as evidenced in studies examining white adipose tissue of obese insulin-resistant mouse models where electron transport chain inactivity and reduced mitochondrial number is observed[12–14]. In addition, human adipocytes from obese subjects have decreased oxygen consumption rates and citrate synthase

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activity[15], while bariatric surgery has been shown to improve a number of different mitochondrial functions as evidenced by gene expression analysis[16]. Mitochondria also alter their morphology as a mechanism for bioenergetic adaption to different metabolic demands[1]. As such, in response to diet-induced obesity, cells favour a fragmented architecture associated with decreased efficiency of ATP production and increased reactive oxygen species (ROS) release[17,18], while in contrast to conditions of obesity during calorie restriction they tend to remain elongated[1]. The changes in mitochondrial dynamics, arising from calorie-restriction, leads to decreased cell oxidative injury, associated with improved insulin sensitivity and longevity[19,20].

The distinct complex roles played by the ER and mitochondria has often led studies to investigate them independently. However, the two organelles are functionally and physically closely interconnected to exchange metabolites, maintain metabolic function and calcium homeostasis, and signal apoptosis[21–24]. Thus, the dysfunction of one organelle can deleteriously affect the other and abnormal ER-mitochondrial cross-talk is intrinsically associated with the pathogenesis of a diverse range of diseases, including metabolic disorders[23,25–27]. Indeed, ER stress has been observed to influence various aspects of mitochondrial form and function, promoting mitochondrial remodelling, depolarisation and ROS production in rodent and *in vitro* human cell models[28–31]. It is therefore, reasonable to assume that ER stress contributes to mitochondrial maladaptation, which may also occur in human adipose tissue, specifically in obesity. Investigation of the interplay between these organelles has hitherto been carried out in hepatocytes[25,29], cancer cells[28,30] and skeletal muscle[32,33]. Limited analysis in murine 3T3-L1 adipocytes has evaluated the effect of ER stress on mitochondrial changes[34], though the impact on mitochondrial respiration and mitochondrial dynamics remains unknown in murine and human adipocytes. This is despite the important role adipocytes play in contributing to the pathogenesis of the metabolic dysfunction[35–38].

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Thus, the aims of these studies were to investigate how ER stress affects mitochondrial form and function in human adipocytes, with a specific focus on mitochondrial respiration, morphology, dynamics and oxidative stress in both an adipocyte cell model and primary human adipocytes, in conditions of obesity.

Materials and methods:

Subjects and sample collection

A female cohort of lean (age: 28.6±(SEM)6yrs; BMI: 20.34±(SEM)0.9 Kg/m^2; n=4) and obese (age: 32.0±5yrs; BMI: 37.58±6.8 Kg/m^2; n=4) adult patients undergoing abdominal elective non-emergency surgery were recruited. All subjects were pre-menopausal, non-diabetic, Caucasian women and any patients who were taking thiazolidinediones and other medication known to affect mitochondrial function were excluded. Each patient provided written, informed consent before these procedures as part of full ethical approval application.

Pre-adipocyte isolation from adipose tissue

Abdominal subcutaneous (AbdSc) adipose tissue was digested as previously described to isolate pre-adipocyte cells[39]. In short, adipose tissue was incubated with collagenase class 1 (2mg/mL; Worthington Biochemical Corporation, Reading, UK) for 30 min, the digest was then filtered through a cotton mesh and centrifuged at 360g for 5 min. The resultant stromal vascular fraction pellet was re-suspended in Dulbecco's modified Eagle's medium with high glucose (DMEM/F12), 10% FBS and 10 μg/mL transferrin. The cells were then maintained in 37°C and 5% CO_2 incubator.

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Proliferation of Chub-S7 cells

Chub-S7 is a cell line derived from subcutaneous abdominal white adipocytes. Similar to primary cell cultures, Chub-S7 cells were maintained in DMEM/F12 supplement with 10% FBS and 10µg/mL transferrin in 37°C and 5% CO_2 incubator.

Human preadipocyte differentiation

Two days post-confluence, the cells were differentiated for four days in DMEM/F12 with 3% FBS and Differentiation Supplement Mix (Promocell, Heidelberg, Germany). On day four, the media was changed to DMEM/F12 with Nutrition Supplement Mix (Promocell) and maintained for 10 days until fully differentiated. After differentiation, cells were allowed to equilibrate in basal media (DMEM/F12 with 0.5% BSA) for 12 hr, before being treated for 24, 48 and 72 hr with DMSO (vehicle control, Sigma-Aldrich, St. Louis, MO), 0.25µg/mL or 0.75µg/mL tunicamycin (Tn; Sigma-Aldrich).

Lipid staining of primary human adipocytes

Lipid staining was performed using a method described by Culling *et al.* and previously utilised during human adipocyte differentiation by McTernan *et al.*[40,41]. Briefly, at regular intervals during differentiation, cells were washed with PBS, fixed with 10% formalin and stained with 2.5% Oil Red O (ORO) for 15 min at room temperature. Cells were then washed with distilled water and viewed under a light microscope to assess lipid accumulation. Propan-2ol was used to elute the ORO and lipid accumulation was quantified by measuring absorbance at 520nM using a spectrophotometer. This method was used to screen primary human pre-adipocytes from lean and obese cohorts in order to select those which showed the least variability between the two groups.

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mRNA transcript quantification and normalisation

RNA isolation was performed using the isolate II RNA Mini Kit (Bioline, Memphis, TN) according to manufacturer's instructions, followed by a DNase digestion step. cDNA was synthesised using reverse transcription reagents (Bioline, London, UK). qPCR was performed with TaqMan probes (18S, Hs03003631_g1; CHOP, Hs00358796_g1; ATF6, Hs00232586_m1; Applied Biosystems, Warrington, UK). Transcript abundance was measured with an Applied Biosystems 7500 Real-Time PCR System with TaqMan universal PCR master mix. All reactions were carried out from an independent study, in triplicate, and multiplexed with the housekeeping gene 18S, to normalise qPCR data. Gene expression was calculated by $2^{-\Delta Ct}$ method.

Genomic DNA (gDNA) transcript quantification and normalisation

Total DNA was isolated from cultured adipocytes with a silica and spin column-based DNA purification kit (DNeasy Blood and Tissue Mini Kit; Qiagen, Crawley, UK) in accordance to the manufacturer's instructions. RNase treatment was performed to eliminate possible RNA contamination. DNA was eluted with 100µL AE buffer and quantified using a spectrophotometer. Relative amounts of mitochondrial DNA copy number were assessed through qPCR in an ABI Prism 7500 thermo cycler (Applied Biosystems, Warrington, UK) with the use of TaqMan Universal PCR Master Mix (Applied Biosystems). Mitochondrial (mtCYB, Hs02596867_s1; mtND1, Hs02596873_s1; mtND5, Hs02596878_g1; Applied Biosystems) and nuclear (18S; Applied Biosystems) gene primers were used to determine relative amounts of mitochondrial to nuclear DNA. Gene expression was calculated by $2^{-\Delta Ct}$ method.

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Protein determination and western blot analysis

For protein analysis, cultured adipocytes from an independent study were lysed in RIPA buffer (Cell Signaling, Denver, MA) supplemented with protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). After harvest, protein concentrations of the cell culture lysates were measured with a Bio-Rad detergent compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA). Western blotting was performed as described previously[42], in brief 20µg protein were loaded onto a denaturing polyacrylamide gel and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with antibodies against OPA1 (1:1000, BD Biosciences, San Jose, CA), p-DRP1 (1:500, Cell Signaling, Danvers, MA), DRP1 (1:1000, Cell Signaling) and MFN2 (1:1000; Abcam, Cambridge, MA), and subsequently with peroxidase-conjugated secondary antibodies. Equal protein loading was confirmed by examining ß-actin (1:5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) protein expression. Proteins were detected using the GeneGnome XRQ chemi-luminescence imaging system (Syngene, Frederick, MD) and band intensities were quantified with ImageQuant TL software (GE Healthcare Life Science, Piscataway, NJ).

Determination of mitochondrial membrane potential (MMP)

The dye tetramethylrhodamine ethyl ester perchlorate (TMRE; Sigma-Aldrich) was used to determine MMP. Chub-S7 cells were grown and differentiated on gelatine-coated 96-well white opaque plates at a density of 10,000 cells/well. Cells were incubated with 300 nM TMRE in serum-free DMEM for 30 min at 37°C. As a positive control for depolarisation, 30 µM FCCP was added to selected cells for 30 min, prior to the TMRE incubation step. Fluorescence intensities (550nM excitation and 590nM emission) were then measured using a PheraStar FS microplate reader (BMG Labtech, Aylesbury, UK). MMP was calculated using the TMRE fluorescence signal relative to the baseline vehicle control.

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Bioluminescent determination of ATP concentrations

Intracellular ATP was measured using the EnzyLight ATP Assay Kit (BioAssay Systems, Hayward, CA) according to manufacturer's instructions. In brief, white opaque 96-well microplates were coated with 0.1% gelatine, on which Chub-S7 cells were cultured. Differentiated adipocytes were pre-treated with DMSO (vehicle control) or tunicamycin (0.25µg/mL and 0.75µg/mL) for 24hr, 48hr and 72hr. On the day of the assay, ATP standards (0 to 30µmoL) were transferred into blank wells in duplicate. At the time of the assay, 95µL assay buffer with 1µL substrate and 1µL ATP enzyme were added to each well containing cells. Luminescence was read on a PheraStar FS microplate reader within 1 min of adding the assay buffer and a standard curve was used to quantify unknown ATP concentrations. This was carried out on three independent occasions.

Oxygen Consumption Rate (OCR) and extracellular acidification rate (ECAR) measurements

OCR was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Santa Clara, CA). Chub-S7 and primary pre-adipocytes were seeded into 0.1% gelatine-coated 24-well Seahorse Microplates (Seahorse Bioscience) at a density of 10,000 cells/well. Cells were differentiated for 14 days as detailed previously, followed by maintenance in basal media for 24 hr. One hr prior to the assay, the media was changed to Seahorse XF media (Seahorse Bioscience). The XF Cell Mito Stress Test was then carried out on three independent occasions using 2µM Oligomycin, 2µM FCCP and 0.5µM Rotenone/Antimycin. FCCP is an uncoupling agent that collapses the proton gradient, oligomycin shuts down ATP synthase and Rotenone/antimycin A inhibit complex I and complex III, respectively. Optimal drug concentrations were determined in preliminary experiments (data not shown). The XF Glycolysis Stress Test was carried out using 10mM Glucose, 1µM Oligomycin and 50mM 2-DeoxyGlucose (2-DG). The concentration of glucose saturates the cells which catabolize it through the glycolytic pathway; oligomycin then shuts down ATP synthase after which 2-DG inhibits glycolysis completely. Values for both assays were normalized to total protein.

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Analysis of mitochondrial morphology through confocal microscopy

Cells were grown on gelatine-coated 35mm glass bottom culture dishes on six independent occasions (MatTek Corportation, Ashland, MA). Treated adipocytes were incubated with 100 nm Mitotracker Green, in HEPES-buffered serum free DMEM (25 mM) for 20 min at 37°C according to the manufacturer's instructions. A Zeiss LSM 510 META microscope (Carl Zeiss GmbH, Vienna, Austria) equipped with a 40 ×/1.4 oil DIC plan-apochromat objective lens was used to view the specimens. Mitotracker Green was excited at 543 nm for imaging. Morphologic assessment of the mitochondrial network was conducted on confocal images using the Mito-Morphology macro for ImageJ (version 1.42) developed by Dagda *et al.*[43].

Endogenous antioxidant and oxidative stress assays

Total reactive oxygen and nitrogen species were evaluated through green fluorescence using OxiSelect *in vitro* ROS/RNS Assay Kit (Cell Biolabs Inc., San Diego, CA). Activity of endogenous antioxidants SOD and catalase was evaluated through a colorimetric method, using OxiSelect Superoxide Dismutase Activity Assay and OxiSelect Catalase Activity Assay Kits (Cell Biolabs Inc.). All assays were carried out according to manufactures instructions.

Statistical Analysis

Significant differences between three or more conditions were assessed by one-way ANOVA; significant differences between two conditions were assessed by a two-tailed Student's *t* test. A result with a *P*-value of ≤ 0.05 was considered statistically significant. In graphs, results are represented as means ± SEM and statistical differences compared to control are indicated with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

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Results

Tunicamycin induced increase of ER stress markers in adipocytes

To investigate the effect of ER stress on mitochondrial form and function, a human adipocytes cell line (Chub-S7) was subjected to increasing doses of tunicamycin - 0.25µg/mL and 0.75µg/mL. To establish UPR activation, key ER stress markers were measured by qPCR. Tunicamycin treatment resulted in a dose dependent increase in mRNA expression of *CHOP* and *ATF6* ($p<0.01$; Figure 1), two transcription factors that play essential roles in the unfolded protein response. Thus, the tunicamycin doses used were appropriate for inducing ER stress in human adipocytes.

ER stress drives an adaptive increase in overall mitochondrial respiration

To explore the possible consequence of ER stress on cellular respiration in human adipocytes, OCR and ECAR were measured in Chub-S7 adipocytes using a Seahorse XF Extracellular Flux Analyser. Under these conditions, maximal respiratory capacity, following injection of FCCP, was higher at all timepoints following tunicamycin treatment (Figure 2a-c). The smallest increase in maximal respiration was observed after 24 hr of incubation with tunicamycin (Figure 2a), while treatment for 48 and 72 hr showed greater, significant increases ($p<0.001$; Figure 2b-c).

A glycolytic stress test indicated that glycolytic capacity, following injection of oligomycin, was significantly higher at most time points in a dose-dependent manner with tunicamycin treatment ($p<0.05$; Figure 2a-c). Consistent with the trend seen with maximal respiratory capacity, the smallest increase in glycolytic capacity was observed after 24 hr of tunicamycin treatment ($p<0.05$; Figure 2a), while 48 hr and 72 hr tunicamycin treatment resulted in greater increases ($p<0.05$; Figure 2e-f).

The spare respiratory capacity (SRC) increased at all time points with tunicamycin, with a significant increase occurring at 72 hr ($p<0.01$; Figure 3a). The largest effect on ATP abundance was observed at 72 hr, with decreased ATP observed in response to both 0.25µg/mL and 0.75µg/mL tunicamycin

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(p<0.05; Figure 3b). Accordingly, tunicamycin-induced ER stress at 48 and 72 hr was associated with significantly decreased mitochondrial efficiency, (p<0.01; Figure 3c), calculated as the ratio of ATP synthesis to oxygen consumed. The impairment of mitochondrial bioenergetics suggests that ER stress leads to an increase in mitochondrial respiration to compensate for the stress the Chub-S7 adipocytes are under, but the cells fail to sufficiently increase ATP to meet the new demands in energy. Moreover, tunicamycin treatment also led to a significant decrease in mitochondrial membrane potential (p<0.05; Figure 3d), as confirmed by TMRE analysis, this reduction was more evident at 24 and 48 hr.

ER stress drives remodelling of mitochondrial network

Mitochondria are often located at intracellular locations of high energy demand and reorganise to meet the metabolic needs of the cells in response to stress. On this basis, the contribution of ER stress to alterations in mitochondrial dynamics in human adipocytes was investigated. In order to detect changes in the morphology of the mitochondrial network, Chub-S7 adipocytes pre-treated with 0.75 μg/mL tunicamycin for 24 hr were imaged with a confocal microscope and four parameters of mitochondrial morphology were quantified: fragmentation, swelling, area and number. It was observed that mitochondria in cells pre-treated with tunicamycin were more fragmented (p<0.01; Figure 4a) and swollen (p<0.01; Figure 4b), and displayed a disorganised morphology compared with the tubular mitochondria of control cells (Figure 4e). In addition to increased fragmentation, a 40% increase in the average area of mitochondria was observed (p<0.05; Figure 4c), which suggests abnormal swelling. There were no significant changes in mitochondrial number observed due to ER stress (Figure 4d).

Based on the aforementioned findings which demonstrated that ER stress induces mitochondrial fragmentation, it was reasoned that this dynamic remodelling occurred due to changes in the core machinery of mitochondrial dynamics which is comprised of three large GTPases that split and fuse the mitochondrial membranes: DRP1, MFN2 and OPA1[44]. All time-points displayed markedly higher levels of DRP1 phosphorylation, the protein responsible for mitochondrial fission (p<0.05; Figure 5a).

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There were no significant changes in MFN2 and OPA1 protein levels at either 24, 48 or 72 hr of tunicamycin treatment (Figure 5b-c).

Mitochondrial content remains unaltered during ER stress

mtDNA copy number was measured as an independent readout of mitochondrial content. mRNA expression of three different genes encoded in the mitochondrial genome, *mtCYB*, *mtND1* and *mtND5*, was detected and the mtDNA/nuclear DNA ratio was calculated. Tunicamycin did not lead to significant changes in mitochondrial copy number although there was a trend towards a decrease (Figure 6a-c).

ER stress increases oxidative stress and reduces antioxidant protection

To elucidate whether ER stress promotes oxidative stress in human adipocytes, Chub-S7 adipocytes were treated with tunicamycin over 72 hr and total ROS and reactive nitrogen species (RNS) were analysed by fluorescence measurements. Abundance of total ROS and RNS rose significantly at 72 hr following both low and high tunicamycin treatment ($p<0.05$; Figure 7a).

Prevention of ROS overproduction via antioxidant protection is a vital matter, to protect against weight gain and insulin resistance. Superoxide dismutase (SOD) an enzyme pivotal in clearing ROS, showed a marked increase in activity with tunicamycin-induced ER stress at most time points ($p<0.05$; Figure 7b). However, the activity of endogenous antioxidant catalase was considerably impaired with ER stress, particularly at 48 hr when tunicamycin treatment resulted in an approximately 30% decrease in catalase ($p<0.05$; Figure 7c). The higher dose of tunicamycin (0.75 μg/mL) also significantly reduced catalase following incubation for 24 ($p<0.05$; Figure 7c). These observations further reflect the capacity of ER stress to negatively influence mitochondrial function in human adipocytes.

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Alterations in mitochondrial respiration are a long-term consequence of obesity

These current studies also sought to monitor the respiratory rates in primary adipocytes isolated from lean and obese age-matched women upon tunicamycin-induced ER stress. The differentiation of primary adipocytes from lean and obese individuals was assessed via Oil Red O staining, utilising adipocyte samples from lean and obese individuals with similar lipid accumulation rates. The bioenergetic function of adipocytes derived from lean participants was consistent with that observed in Chub-S7 cells after 24 hr of tunicamycin treatment: the spare respiratory capacity (SRC) increased with tunicamycin treatment, with a significant increase observed with 0.75μg/mL tunicamycin (p<0.001; Fig. 8a). In contrast, adipocytes from obese individuals showed an inability to trigger an adaptive response to tunicamycin-induced ER stress, as evident by the lack of increase in SRC after exposure to 0.25μg/mL and 0.75μg/mL tunicamycin. These findings may also be attributable to the high SRC in the control cells of obese individuals (Fig. 8a), which suggests that these subjects are already under ER stress resulting in matched SRC of the untreated adipocytes with the tunicamycin-induced stressed adipocytes.

Additionally, our findings revealed that the basal respiratory capacity in adipocytes from obese individuals was greatly diminished compared with adipocytes derived from lean individuals (p<0.01; Fig. 8b). Obesity also mediated a modest decrease in ATP production compared with lean participants, although this did not reach significance (Fig. 8c). Taken together, these findings strongly suggest that the initial adaptive response to ER stress is short-term, whilst chronic ER stress diminishes respiratory capacity and the ability of mitochondria to launch an adaptive response.

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Discussion

In this study, it was hypothesised that ER stress leads to mitochondrial damage in human adipocytes, exacerbated by conditions of obesity. To investigate this, Chub-S7 adipocytes were used as a cell model, and primary human adipocytes were utilised to determine how ER stress may impact on mitochondrial function. From these investigations our studies suggest that ER stress mediates mitochondrial dysfunction in human adipocytes, exacerbated in obesity, as evidenced by: (1) diminished mitochondrial efficiency of Chub-S7 adipocytes which continues to drop with prolonged exposure to ER stress, paired with increased spare respiratory capacity; (2) mitochondrial function being impaired with increased adiposity (as evidenced by the diminished ability of the obese adipocytes' SRC to respond to ER stress); and (3) ER stress directly generating fragmented mitochondria as visualised by imaging mitochondrial fragmentation and through changes in p-DRP1 protein expression, leading to oxidative stress.

Functional assessment of ER-mediated mitochondrial damage was undertaken by assessing real time measurement of oxygen consumption in Chub-S7 adipocytes and primary adipocytes. Chub-S7 cells were used as a cell model to monitor the direct influence of ER stress on mitochondrial function, independent of patient variability. It was noted that Chub-S7 adipocytes exposed to ER stress exhibited increased spare respiratory capacity (SRC), which is the amount of extra ATP that can be produced by oxidative phosphorylation in case of a sudden increase in energy demand. This increase in the SRC may be crucial for mitochondria to be able to function above their full respiratory potential in response to ER stress, in order to match the additional energy demands required to re-establish protein homeostasis. In the realm of chronic overnutrition, the importance of maintaining efficient protein folding in adipocytes is tied to the notion that the ER is directly involved with lipid homeostasis[45]. However, under the obesity condition, the excessive accumulation of lipids, and thus lipotoxicity may result[45]. Accordingly, these studies monitored the respiratory rates in lean and obese subjects, noting that previous work has demonstrated that both ER stress and mitochondrial dysfunction are increased in obese individuals compared to lean[8,15,46]. Whilst the differentiation of

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cells isolated from lean and obese subjects can differ significantly, preadipocytes from different participants (both lean and obese) were screened, reducing this variability. While adipocytes from lean individuals showed the ability to compensate for tunicamycin-induced ER stress by increased SRC, obese individuals were unable to respond to tunicamycin-induced ER stress. In fact, adipocytes from obese participants also exhibited significantly diminished basal respiration, which is in line with previous reports in human primary adipocytes[15,47]. This reduced basal respiration could occur due to a decrease in demand for ATP, either via translation attenuation during ER stress reducing the amount of ATP-dependent processes occurring, or as an adaptive response to limit the increase in fat mass. This second theory is supported by the reduction in expression of acetyl-CoA carboxylase, essential in fatty acid synthesis, as well as reduced *de novo* lipogenesis in obesity[48,49]. Additionally, this reduced basal respiration in adipocytes from obese participants suggests that mitochondria are able to manage oxygen consumption to mitigate the impact of acute ER stress (as observed in Chub-S7 cells), but long-term obesity leads to mitochondrial damage and thus metabolic maladaptation. This reduced respiration in obesity may lead to decreased substrate oxidation, most notably diminished oxidation of fatty acids, leading to ectopic lipid accumulation and subsequently giving rise to insulin resistance and other comorbidities[47]. OCR measurement in Chub-S7 adipocytes identified that whilst maximal respiration remained raised over time in response to tunicamycin treatment, in control cells the maximal respiration declined over time whilst staying within an acceptable OCR range for such untreated cells. This decline in OCR in control cells may have arisen as the adipocytes utilise the remaining components from the differentiation media in the acute phase (24 hr), despite a wash out period being included. This effect may also have arisen in the tunicamycin-treated cells, although masked by the treatment. This did not change the continued impact of tunicamycin on maximal respiration, or override the effect on OCR in Chub-S7 adipocytes.

It was also observed that chronic ER stress in Chub-S7 adipocytes decreased mitochondrial efficiency, while acute ER stress had no evident effects. Glycolysis, the less efficient metabolic process for ATP synthesis was therefore investigated. Respiratory studies in Chub-S7 adipocytes revealed that following tunicamycin treatment, the cells had an increased dependence on glycolysis

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(higher dependence on glucose). This was true for both acute (24 hr) and chronic (72 hr) treatments, indicating that the cells are over-compensating for the ER stress by increasing overall respiration. Nonetheless, the efficiency of ATP production eventually plummets despite these efforts of the cell. As a result inefficient mitochondria in obesity may be unable to meet the energy demand required for protein folding[50–52]. Many proteins required for lipid handling are processed in the ER and if these proteins are misfolded it may, in the long-term, lead to ectopic lipid deposition[45]. As such if adipose tissue is not able to buffer lipids, lipids will spill-over into the bloodstream resulting in lipotoxicity in other cell types, an underlying cause of obesity-associated insulin resistance and atherosclerosis[38,53]. ER stress-induced mitochondrial dysfunction may therefore be another factor that promotes ectopic fat deposition in non-adipose tissues.

The effect of ER stress on metabolism may arise from the influence on multiple pathways and structural components of mitochondria. Previous studies have shown that a response to changes in energy supply and demand results in mitochondria remodelling their architecture[1]. This change in modelling has been identified in mice with genetically induced obesity or on a high fat diet, which display increased levels of mitochondrial fission proteins (DRP1 and Fis1) in skeletal muscle[17]. In line with these insights our current human adipocyte studies demonstrated that ER stress induced dynamic remodelling of the mitochondrial network in Chub-S7 adipocytes, evident most notably by increased fragmentation and swelling of mitochondria. Additionally, active image analysis of mitochondrial dynamics in Chub-S7 adipocytes, appear to affirm changes observed in Hela cells which reported that following 24 hr of treatment with thapsigargin (an ER stressor) the population of fragmented mitochondria increased[54]. As such, these findings indicate that chronic ER stress is instrumental as a pathway contributing to mitochondrial fragmentation in human adipocytes. These findings have implications on metabolic health in obesity, as mitochondrial fragmentation is known to contribute to the development of type 2 diabetes mellitus[17,54].

By analysing the protein expression of mitochondrial fission/fusion machinery in Chub-S7 adipocytes, the mechanism by which ER stress induces fragmentation, as evidenced by confocal

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imaging, could be determined. The protein DRP1, a GTPase that catalyses the process of mitochondrial fission, was investigated. The findings of these studies revealed upregulation of DRP1 phosphorylation following tunicamycin incubation, suggesting enhanced mitochondrial fission. This suggestion arises as a number of previous non-adipocyte studies have demonstrated that phosphorylation of DRP1 promotes DRP1 translocation to the mitochondria resulting in mitochondrial fission[55–57]. As such these current studies may therefore indicate that the increased DRP1 phosphorylation in Chub-S7 adipocytes, in response to ER stress, promotes mitochondrial fragmentation. Of note, the functional consequence of DRP1 phosphorylation is still somewhat disputed, as some contradictory studies (non-adipocyte) have reported that phosphorylation of DRP1 resulted in elongated mitochondria, rather than stimulating mitochondrial fission[58,59]. This conflict may merely suggest that the changes in DRP1 phosphorylation are cell type- and stimulus-dependent, however in the case of human adipocytes, phosphorylation of DRP1 appears to result in mitochondrial fragmentation via ER stress.

Fragmented mitochondria are also a source of oxidative stress, as previously observed in rat myoblasts and human endothelial cells[60,61]. In addition, silencing DRP1 has been shown to alleviate mitochondrial fission leading to decreased ROS generation[60], suggesting that fission plays an indispensable role in mitochondrial-mediated oxidative stress. Given these findings, oxidative stress was measured following exposure to tunicamycin. This study showed that ER stress initiates ROS production in Chub-S7 adipocytes and reduces the synthesis of catalase, an antioxidant enzyme essential for neutralising these free radicals. ER stress induced-mitochondrial fragmentation may therefore be a causal factor implicated in increased oxidative stress, a major cause of clinical complications associated with obesity[62]. Oxidative stress itself also increases the production of misfolded proteins, causing aberrant mitochondria morphology, which, in turn, further exacerbates oxidative stress in a self-perpetuating vicious cycle[38]. Prevention of ROS accumulation is therefore important to alleviate downstream metabolic dysfunction. Targeting ROS by reducing mitochondrial fragmentation may be a viable option. Additionally, antioxidants such as α-lipoic[38] acid or pharmacological compounds including thiazolidinediones[63] and metformin[64] also lead to metabolic

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improvements by reducing ROS production, decreasing the accumulation of toxic lipid metabolites and enhancing mitochondrial biogenesis; all protective factors against weight gain and insulin resistance.

On the basis of the findings shown here, we propose that in human adipocytes excessive ER stress is a precursor to drive mitochondrial dysfunction in obesity that may contribute to the development of metabolic pathologies such as dyslipidaemia and ectopic fat deposition in type 2 diabetes mellitus. Treatments such as salicylate may offer new ways to reduce metabolic pathologies mitigating ER stress, to improve cellular health[65,66].Therefore, reducing nutrient stress or developing therapies that mitigate ER stress in adipocytes may be a useful targeted approach for the treatment of chronic ER stress and mitochondrial dysfunction in metabolic disease.

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Figures Legends:

Figure 1: Effect of tunicamycin concentrations on ER stress markers in Chub-S7 adipocytes. (a) CHOP mRNA expression levels; (b) ATF6 mRNA expression levels; with tunicamycin (0.25µg/mL or 0.75µg/mL) for 24 hr. Error bars represent standard error of the mean. Control vs treatments (one-way ANOVA, n=4): ** $p < 0.01$, *** $p < 0.001$.

Figure 2: Effect of tunicamycin on mitochondrial respiration in Chub-S7 adipocytes. The Seahorse +XF Cell Mito Stress Test was performed with Chub-S7 adipocytes and the OCR was measured following (a) 24, (b) 48 or (c) 72-hr treatment with varying doses of tunicamycin (0.25µg/mL or 0.75µg/mL). The Seahorse XF glycolytic stress test was also performed on Chub-S7 adipocytes, with ECAR measurements taken at (d) 24, (e) 48 or (f) 72 hr following treatment with tunicamycin. Dotted lines indicate injections into media of the specific stressors - oligomycin (Oligo), carbonyl cyanite-4 (trifluoromethoxy) phenylhydrazone (FCCP) and Rotenone/Antimycin A (Rot/AA) for the Cell Mito Stress Test; glucose, oligo and 2-deoxyglucose (2-DG) for the Glycolytic Stress Test. One-way ANOVA (n=3) was carried out - control vs 0.25µg/mL tunicamycin: $^{\tau}p < 0.05$, control vs 0.75µg/mL tunicamycin: $^{*}p < 0.05$, $^{**}p < 0.01$.

Figure 3: Assessment of mitochondrial energetics in Chub-S7 adipocytes.

(a) Spare respiratory capacity (SRC % = (Maximal Respiration)/(Basal Respiration) x 100), (b) ATP abundance (calculated from EnzyLight ATP Assay Kit using ATP standard curve), (c) mitochondrial efficiency (expressed as the ratio of ATP synthesised and oxygen consumed) and (d) MMP (with FCCP used as a positive control for depolarisation) were measured on following incubation with tunicamycin (0.25µg/mL and 0.75µg/mL) for 24, 48 and 72 hr. Data are expressed as mean ± standard

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error of the mean. Control vs treatments (one-way ANOVA, n=3): $*p < 0.05$, $** p < 0.01$, $***p < 0.001$.

Figure 4: Effect of ER stress on mitochondrial morphology in Chub-S7 adipocytes. Confocal images were taken and analysed to determine (a) mitochondrial fragmentation, (b) mitochondrial swelling, (c) mitochondrial area and (d) number of mitochondria. (e) Representative live confocal images of MitoTracker Green-stained Chub-S7 cells after 24-hr treatment with 0.75µg/mL tunicamycin, taken with a confocal microscopy at 40x magnification. The inset shows a magnification of part of the image indicated by the asterisk. Scale bars, 10µm. Control vs treatment (two-tailed Student's T-test, n=6): * $p < 0.05$, ** $p < 0.01$. n.s. = not significant.

Figure 5: Regulation of mitochondrial dynamic proteins through ER stress in Chub-S7 adipocytes. Representative Western blot images and protein quantification of (a) p-DRP1/DRP1, (b) L-OPA1 and (c) MFN2 using ImageQuant TL are shown. Control vs treatments (one-way ANOVA, n=3): ** $p < 0.01$, *** $p < 0.001$.

Figure 6: Effect of ER stress on mitochondrial content in Chub-S7 adipocytes. Mitochondrial DNA copy number in human adipocytes following a 24, 48 or 72-hr incubation with 0.25 µg/mL and 0.75 µg/mL tunicamycin was quantified by measuring the mRNA expression of mitochondrial encoded genes (a) *ND5*, (b) *ND1* and (c) *CYB* over *18S*, a nuclear encoded gene. One-way ANOVA was carried out (n=4), however no differences were significant.

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Figure 7: Effect of tunicamycin induced ER stress on oxidative stress and endogenous antioxidant activity. (a) Total reactive oxygen (ROS) and nitrogen species (RNS). (b) Catalase activity. (c) Superoxide dismutase 2 (SOD2) activity in human adipocytes (Chub-S7 cells) following 24, 48 and 72-hr incubation with 0.25 µg/mL or 0.75 µg/mL tunicamycin. Bars represent standard error of the mean. Control vs treatments (one-way ANOVA, n=4): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8: Key parameters of respiratory control in lean and obese adipocytes. A mitochondrial stress test was conducted using primary adipocytes from four lean (d-g) and four obese (h-k) patients following treatment for 24 hr with 0.25µg/mL and 0.75µg/mL tunicamycin. The parameters determined were (a) spare respiratory capacity, (b) basal respiration and (c) ATP production, calculated by taking the lowest rate after oligomycin injection from the basal rate. Boxplots depict grouped patients with n = 4 in each group in triplicate. Data are shown as median and interquartile ranges, percentile ranges are 10-90%. OCR values were normalised to total protein to account for inter-well cell number variability. Control vs treatments (one-way ANOVA, n=3): * $p < 0.05$, **<0.01, *** $p < 0.001$.

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Figure 1:

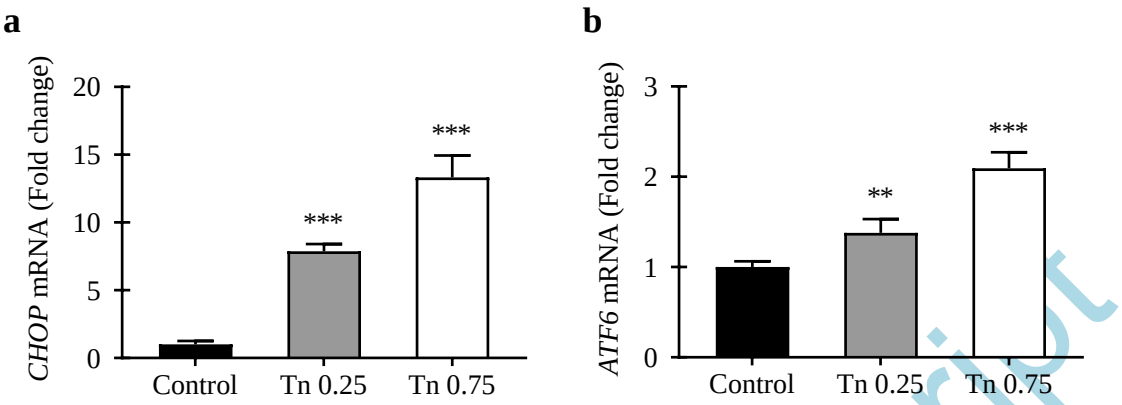

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Figure 2:

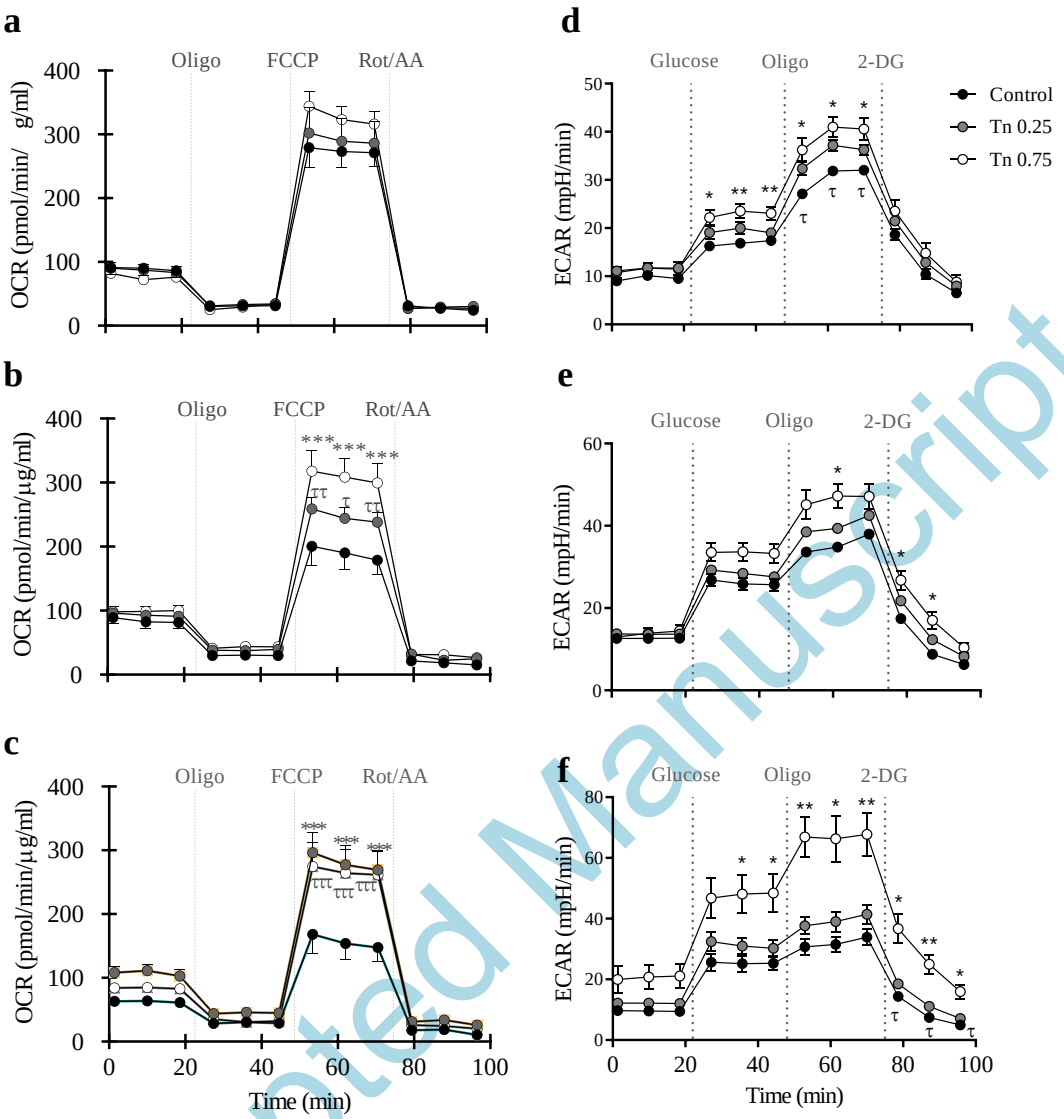

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Figure 3:

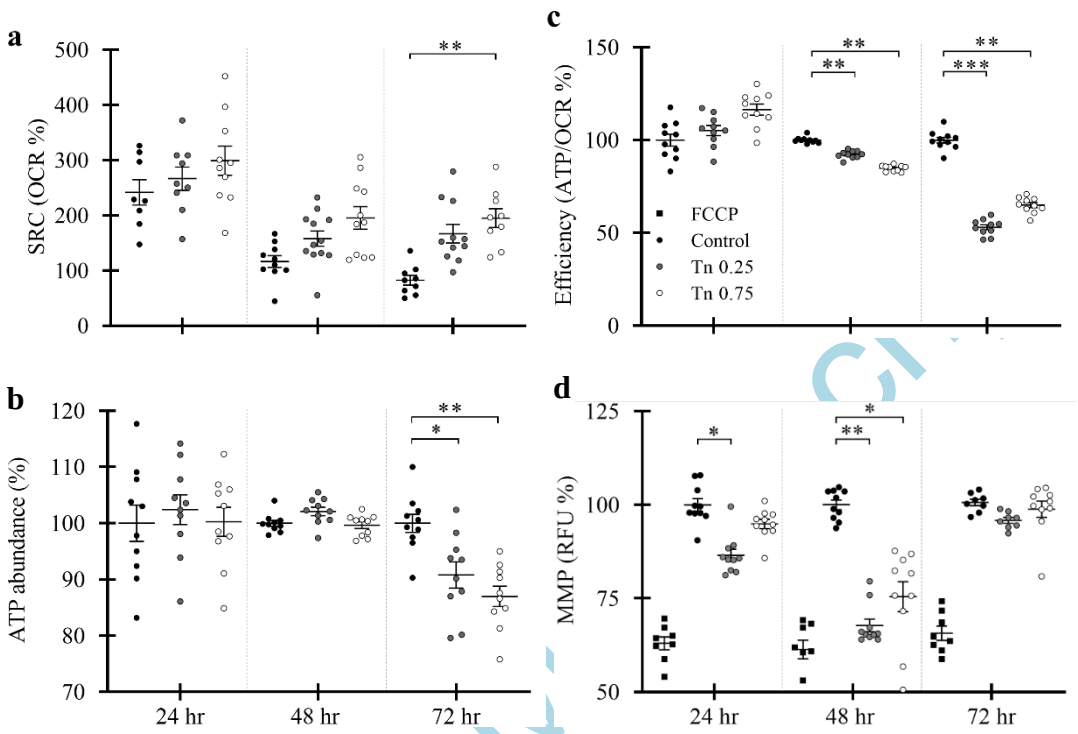

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Figure 4:

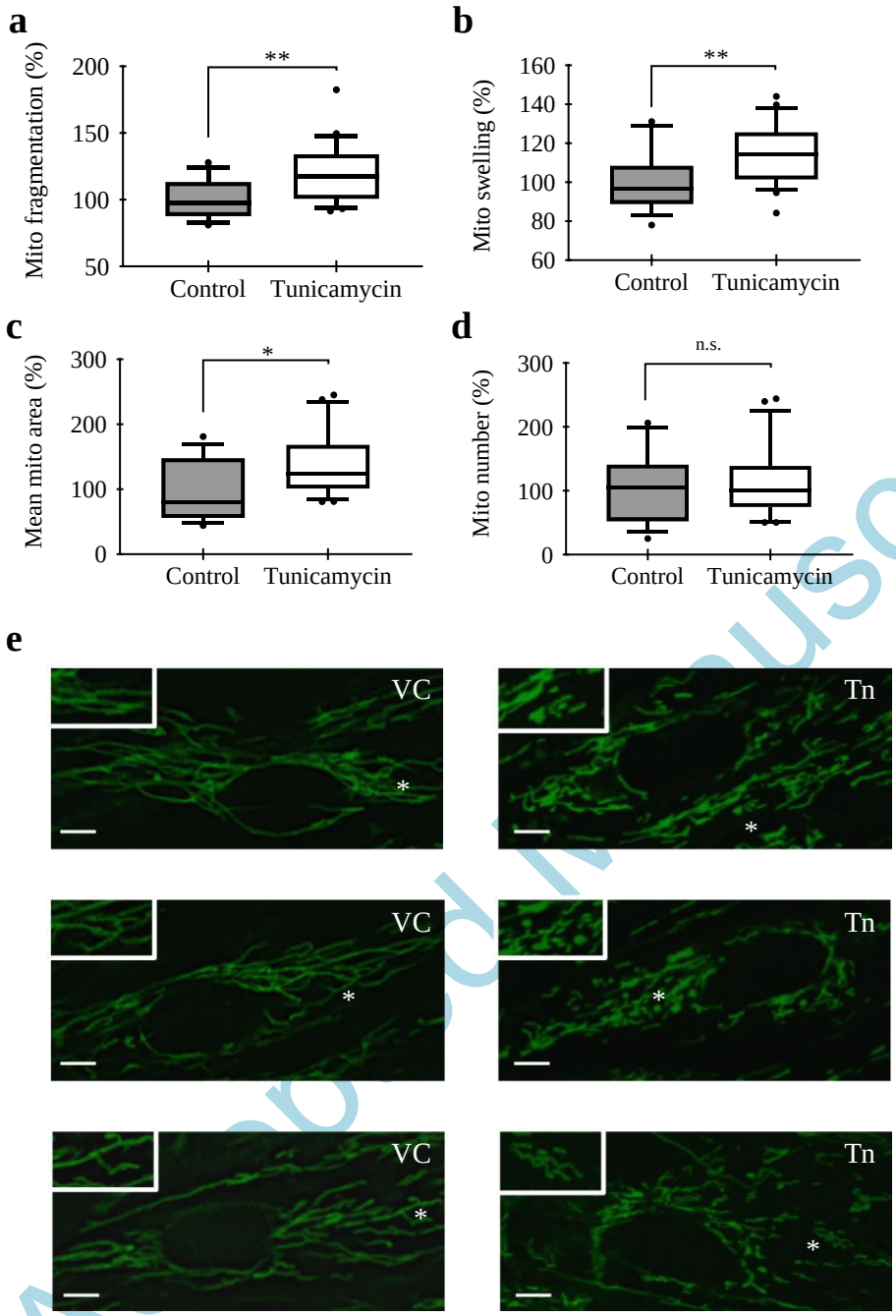

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Figure 5:

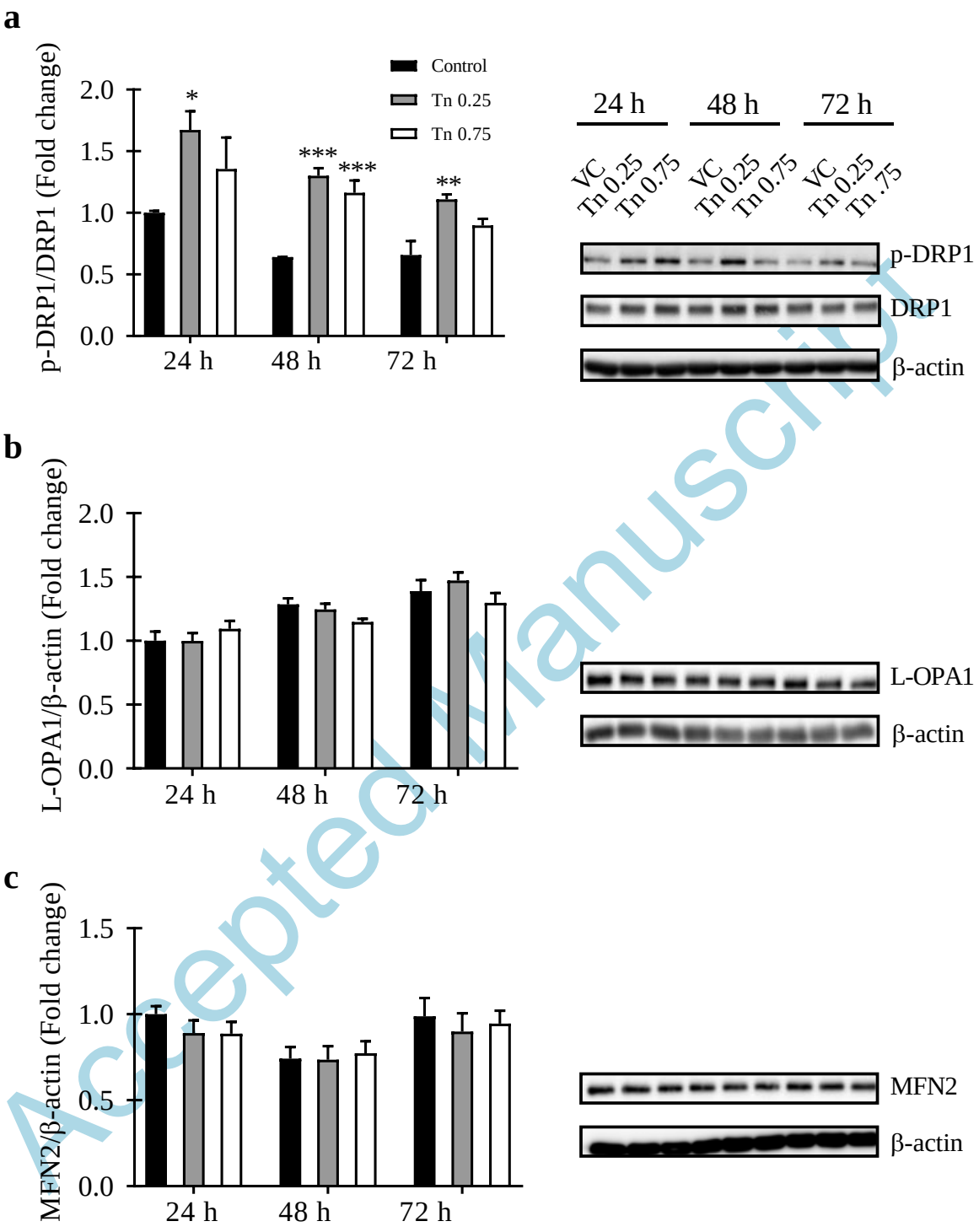

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Figure 6:

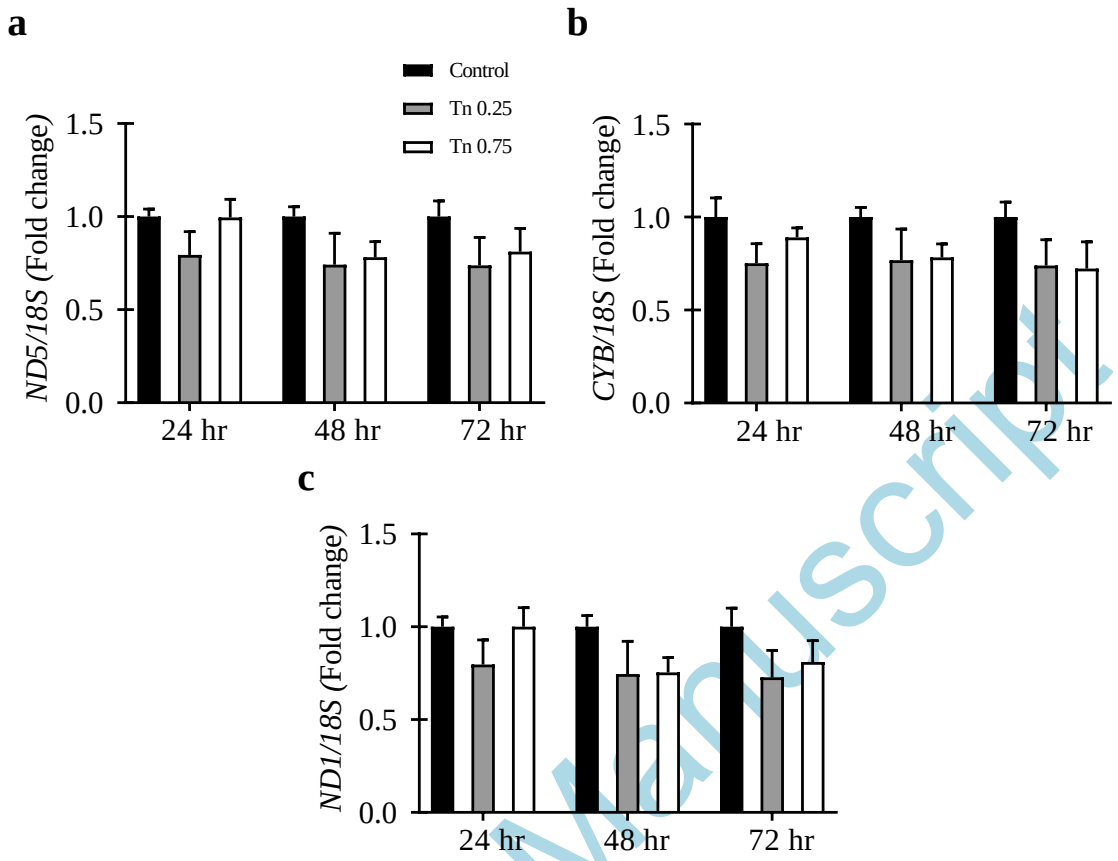

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Figure 7:

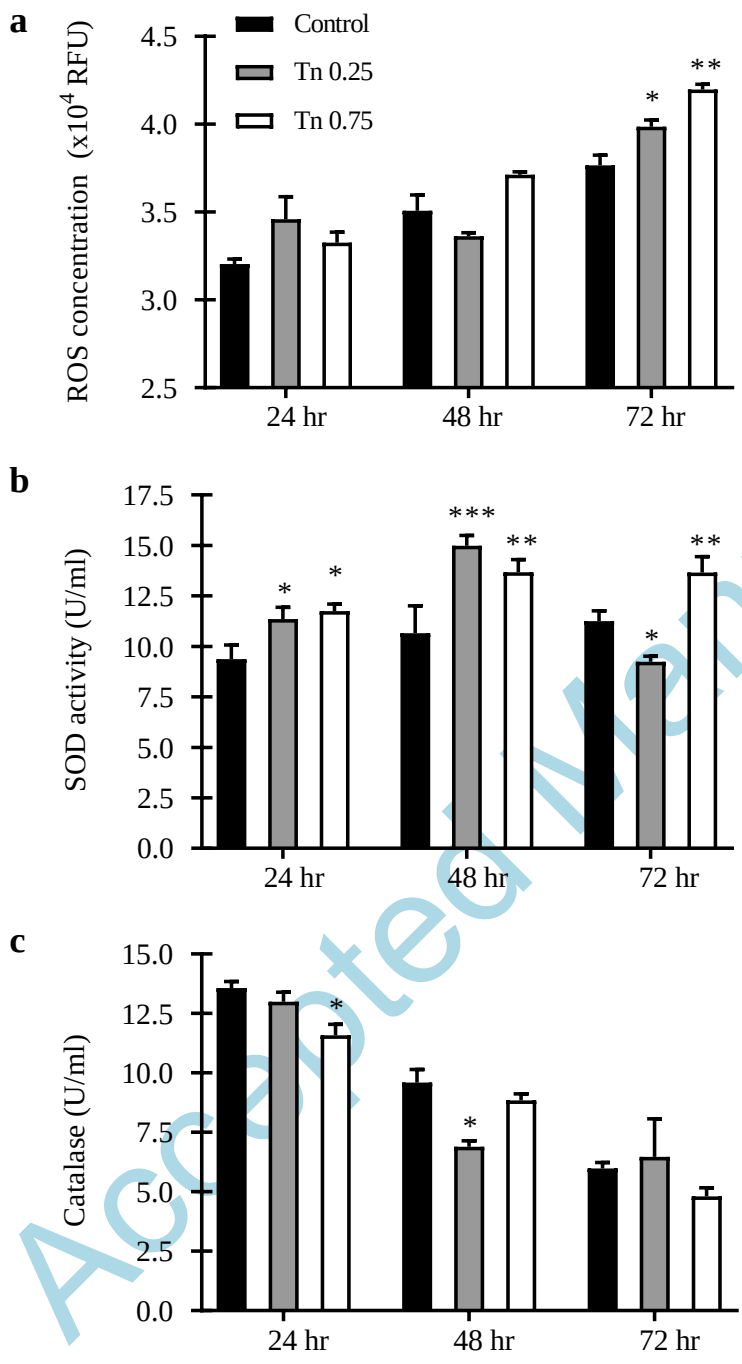

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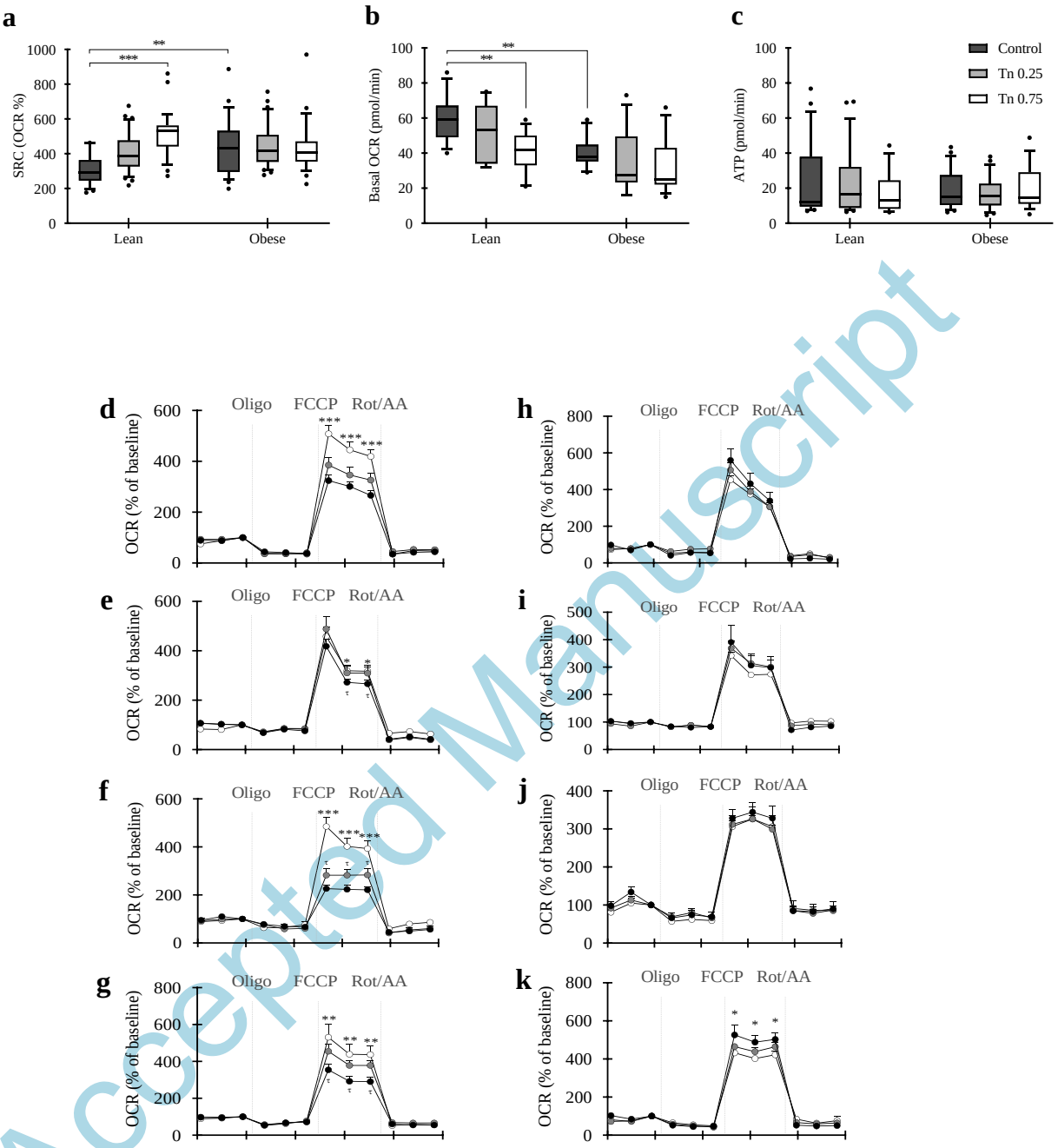
Figure 8:



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