Differential Effects of Metformin and/or Nox1/Nox4 Inhibition upon Oxidative Stress Signaling in mouse skeletal C2C12 Myoblasts Under Diabetic-Mimic Conditions

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Background:

Type 2 Diabetes Mellitus (T2DM) is a chronic, multisystemic, metabolic disorder characterized by elevated blood sugar levels, presenting significant public health challenges. T2DM leads to complications, including coronary heart disease, kidney disease, retinopathy, and neuropathy (Tomic, Shaw, and Magliano, 2022). Individuals with T2DM face a greater risk of sarcopenia, characterized by muscle loss, due to factors such as glucose toxicity, insulin resistance, and oxidative stress (Teng and Huang, 2019). In elderly T2DM patients, impaired insulin signaling disrupts processes like autophagy, increases muscle protein degradation, and causes mitochondrial dysfunction, resulting in muscle mass loss and reduced mobility (Bouchi et al., 2017). Elevated glucose concentrations further negatively affect satellite cell (muscle-specific stem cells) proliferation and self-renewal, impairing the muscle's ability to regenerate after injury and maintain its mass (Furuichi et al., 2021). T2DM primarily stems from insulin resistance (IR) and pancreatic β-cell dysfunction, exacerbated by high glucose levels. IR arises from insulin signaling disruptions (Galicia-Garcia et al., 2020), often linked to oxidative stress signalling driven by Reactive Oxygen Species (ROS), primarily produced by NADPH oxidases (NOXs). Angiotensin II (AngII) aggravates T2DM by triggering NOX-mediated ROS production (Cat et al., 2013). Metformin, the primary antidiabetic medication, is recommended alongside lifestyle modifications. It effectively lowers blood glucose levels, modulates redox signaling pathways, reduces ROS in various cell types, enhances antioxidant capacity, and mitigates oxidative stress (Apostolova et al., 2020). Besides its anti-oxidant effects, recently it has been demonstrated that metformin has an impact on skeletal muscle differentiation/regeneration such as i. involved in the repairing of myofilament damage in skeletal muscle fibres in mouse models of Duchenne muscular dystrophy (Dong et al., 2021), ii. delaying skeletal muscle regeneration in cardiotoxin-induced skeletal muscle damage in mice (Pavlidou et al., 2017) and iii. repairing mitochondrial dysfunction in skeletal muscles of diabetic rats (Kane et al. 2010).

Aims:

Even though the effects of metformin upon skeletal muscle regeneration have been previously documented, the role of metformin in AngII-induced oxidative stress signaling in mouse skeletal myoblasts (undifferentiated skeletal muscle cells) grown in diabetic-mimic conditions is not well understood. Therefore, in this investigation, we wanted to examine whether oxidative stress signaling is impaired in AngII-stimulated skeletal muscle myoblasts grown in diabetic-mimic conditions and whether metformin and/or NOX inhibition can restore it. Additionally, we wanted to examine whether metformin's anti-oxidant effects are through Nox1 or Nox4 activation in mouse skeletal myoblasts (C2C12). Therefore, in this study, we examined the impact of AngII and metformin, alone and combined with Nox inhibitors, on oxidative stress signaling in C2C12 cells under diabetic-mimic conditions. Specifically, the objectives were:

Aim 1: To investigate the effects of varying concentrations of metformin upon ROS levels in AngII-stimulated C2C12 myoblasts grown in diabetic-mimic conditions.

Aim 2: To investigate the differential effects of NOX1/NOX4 inhibition, metformin and combination of metformin and NOX inhibitors upon ROS levels in C2C12 cells grown in diabetic-mimic conditions.

Aim 3: To examine the differential effects of NOX1/NOX4 inhibition, metformin and their combination upon NOX4 protein expression in C2C12 cells grown in diabetic-mimic conditions.

Methods:

Cell Culture and Treatments

Immortalised mouse skeletal myoblasts (C2C12) were grown in 10% fetal calf serum (FCS) in 1xDMEM (containing 5.4mM glucose, NG), and supplemented with penicillin, streptomycin, and amphotericin B (P/S). When cells were ready to set-up for the experiments, cells were seeded either in 96-well black plates with clear bottom (H_2DCFDA assay) or 6-cm petri-dishes (Amplex Red, Western blotting). The following day, cells were grown either in 10% FCS in 1xDMEM with NG or high glucose (HG, 25 mM glucose) for three days. After three days, cells were serum deprived by replacing the media with 1xDMEM with NG or HG and after 24 hrs, the cells were treated with 0.05% DMSO (Vehicle control) or 10 μM GKT136901(GKT, a NOX1/NOX4 inhibitor) or 1 μM ML171 (NOX1 inhibitor) or metformin or combination of metformin with GKT or ML171 and stimulated with or without 100 nM Angiotensin II (AngII) for 24 hrs. Then cells were prepared either for the ROS assays measurements or western-blotting.

H_2DCFDA Assay: A general ROS measurement assay

At the end of each treatment, cells were loaded with 20 μM of the cell-permeant unfluorescent H_2DCFDA probe (Thermofisher) for 45 mins and incubated at 37^0C in a 5% CO_2 incubator. Then the oxidized DCF fluorescence (reduced form of fluorescein by ROS) was measured using a fluorescence plate reader.

Amplex Red Assay: Measurement of Hydrogen peroxide (H_2O_2) generation

H_2O_2 generation in protein extracts was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit according to manufacturer's instructions (Life Technologies).

Western-Blotting (WB)

Protein lysates (20 μg) were separated in 12% SDS-polyacrylamide gels, transferred onto a nitrocellulose membrane and probed with primary antibodies for total NOX4 (Abcam, ab133303) and β-actin (Abcam, ab8224) was used as a loading control. Horseradish peroxidase-conjugated secondary antibodies were visualized by a chemiluminescent imager and densitometry analysis was performed by image J.

A) **B)**

Figure 1: (A) Protein concentration determined by the Pierce-BCA assay. (B) Nitrocellulose membranes stained with Ponceau-S, a reversible staining of proteins.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. All experiments were performed in triplicates (N=3). Data is presented as mean+/-SEM. Statistical comparisons were made with one-way ANOVA analysis and pair-wise comparisons with two-tailed Student's t-test with Welch's correction for unequal variances. $P<0.05$ was considered statistically significant.

Results:

Effects of varying concentrations of metformin upon ROS levels in AngII-stimulated C2C12 Myoblasts grown in diabetic-mimic conditions

This investigation explored the effects of varying concentrations of metformin upon ROS levels in AngII-stimulated C2C12 cells grown in NG or HG conditions. As shown in Figure 2, AngII-stimulated cells grown in HG exhibited higher ROS levels compared to cells grown in NG. Most metformin concentrations had limited effects in unstimulated NG or AngII-stimulated NG cells. However, low (0.010mM) and high (10mM) metformin concentrations slightly increased and significantly reduced ROS respectively in unstimulated NG cells ($P<0.05$). Most metformin concentrations led to a slight ROS reduction in HG, with 0.100mM being significant. Conversely, in HG-AngII cells, most metformin concentrations significantly increased ROS with the highest at 0.010 and10 mM ($P<0.05$). No significant ROS changes were observed with varied AngII concentrations (data not shown). Subsequent experiments focused on NOX1/NOX4 inhibition and metformin's effects upon ROS and NOX4 protein expression.

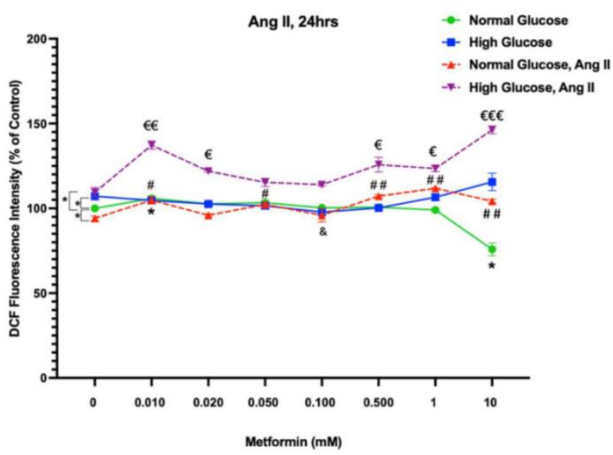
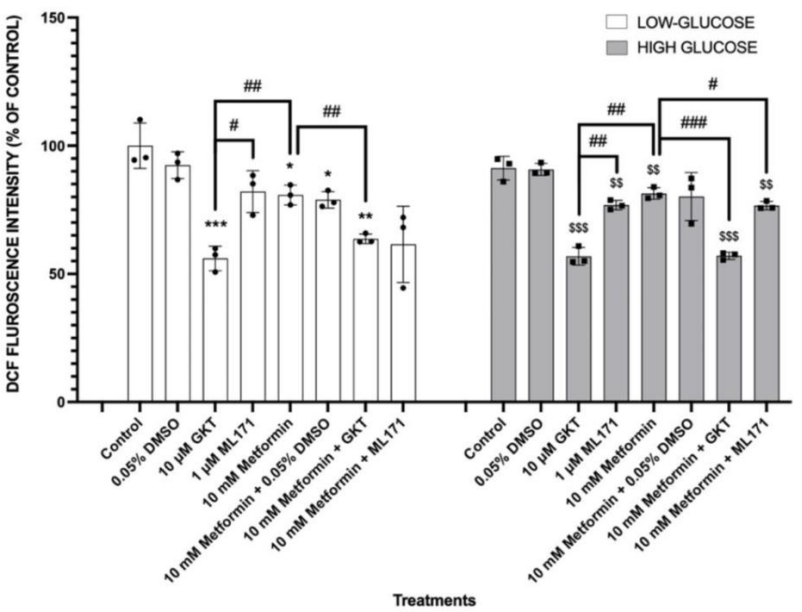


Figure 2. **Effects of varying concentrations of Metformin upon ROS levels in AngII-stimulated C2C12 myoblasts**. C2C12 cells were grown in either NG or HG conditions for three days and underwent overnight serum deprivation. Subsequently, cells were treated with varying metformin concentrations as indicated and stimulated with or without AngII for 24hrs. ROS levels were evaluated using the H_2DCFDA assay. The data is presented as the % change relative to the control (C2C12 cells grown in NG and set as 100%). Pairwise comparisons were performed using unpaired 2-tailed t-test with Welch's correction. $P<0.05$ was considered statistically significant. *$P<0.05$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. AngII in NG; $^{\&}P<0.05$ vs. HG; $^{€}P<0.05$, $^{€€}P<0.01$, $^{€€€}P<0.001$ vs AngII in HG.

Effects of metformin and/or NOX1/NOX4 inhibition upon ROS levels in C2C12 Myoblasts grown in diabetic-mimic conditions

To examine the effects of metformin and/or NOX1/NOX4 inhibition upon ROS levels in C2C12 myoblasts grown in NG or HG, cells were treated either with metformin, GKT, ML171 or combination of metformin with GKT or ML171 for 24 hrs and general ROS and H_20_2 levels were measured by the H_2DCFDA and Amplex Red assays respectively. As shown in Figure 3A, GKT, metformin and combination of metformin with GKT significantly decreased general ROS levels in cells grown either in NG or HG compared to untreated cells grown either in NG or HG ($P<0.05$). ML171 and combination of metformin with ML171 significantly decreased ROS levels in cells grown in HG compared to untreated cells grown in HG ($P<0.05$). Among the different treatments, GKT was the most effective one to significantly decrease general ROS levels in cells grown either in NG or HG indicating that ROS production in these cells is mainly NOX4 dependent. Amplex Red assay showed the following results: i. HG significantly increased H_20_2 levels compared to cells grown in NG, ii. Combination treatment of metformin and GKT significantly increased H_20_2 levels in cells grown in NG compared to untreated cells grown in NG and iii. GKT significantly decreased HG-induced H_20_2 levels in C2C12 myoblasts, indicating that HG-induced H_20_2 levels is mainly NOX4 dependent (Figure 3B, $P<0.05$).

A)



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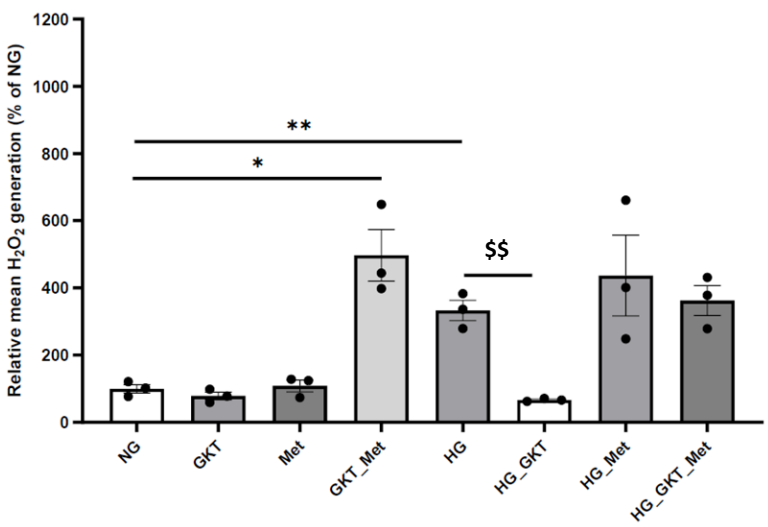


Figure 3. Effects of metformin and NOX1/NOX4 inhibition upon ROS generation in C2C12 myoblasts grown in normal or high glucose conditions. Serum deprived C2C12 myoblasts grown in NG or HG were treated either with 0.05% DMSO or 10 µM GKT136901 (GKT) or 1 µM ML171 or 10 mM metformin (Met), or combinations of Met with GKT or ML171 for 24 hours. General ROS levels were measured using the H_2DCFDA assay (A) and H_20_2 levels were measured using the Amplex Red assay (B). The results are presented as the mean ± SEM (N=3). Data is presented as the % change relative either to the control (cells grown in NG) or vehicle control (cells grown in NG and treated with 0.05% DMSO). The control or vehicle controls were set to 100%. P<0.05 was considered statistically significant. *P<0.05, **P<0.01, ***P<0.001 vs 0.05% DMSO in NG; $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 vs 0.05% DMSO in HG; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 for comparisons among different treatment groups.

Effects of metformin and/or NOX1/NOX4 inhibition upon NOX4 protein expression in C2C12 Myoblasts grown in diabetic-mimic conditions

To examine the effects of metformin and/or NOX1/NOX4 inhibition upon NOX4 protein expression in C2C12 myoblasts grown in diabetic-mimic conditions, cells grown in NG or HG were treated with either 0.05% DMSO or 10µM GKT or 1mM Metformin or their combination for 24 hrs and protein expression of NOX4 isoforms (72kDa and 66kDa) was examined by western-blotting (WB). In NG, GKT, Met, and Met-GKT combinations led to similar non-significant decreases in Nox4_66kDa isoform expression (Figure 4). Metformin significantly reduced Nox4_72kDa isoform expression in NG (P<0.05), while in HG, it slightly decreased without reaching statistical significance. GKT and Met-GKT in HG increased Nox4_72kDa isoform protein expression, though not significantly.

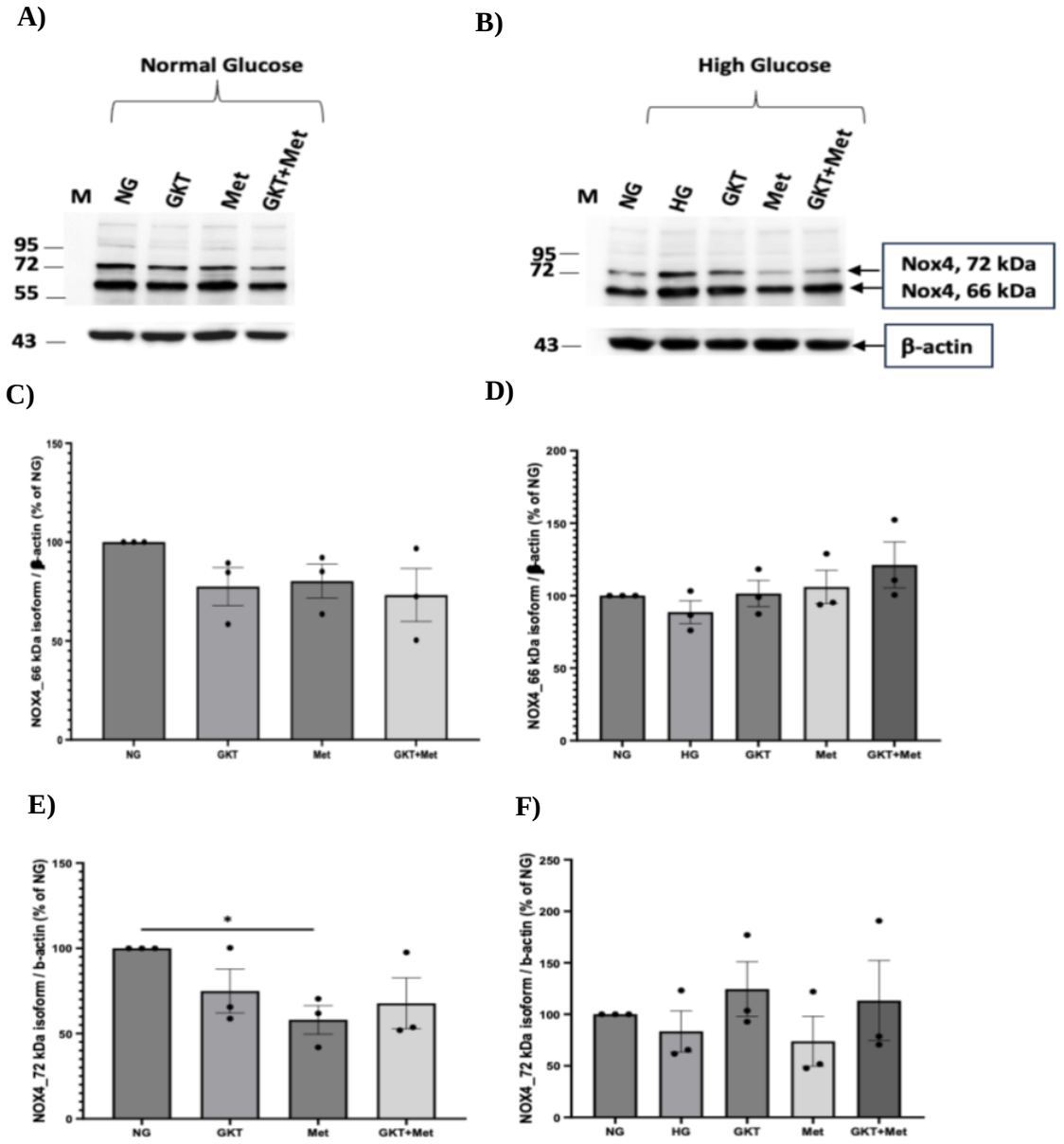


Figure 4: **Effects of metformin and/or NOX1/NOX4 inhibition upon protein expression of total NOX4 in mouse skeletal myoblasts (C2C12) grown either in normal or high glucose conditions**. Serum-deprived C2C12 cells grown in NG or HG were subjected to treatments, including 10 µM GKT136901 (GKT), 1 mM Metformin (Met), or a combination of Met with GKT, for 24 hrs. Following each treatment, cell lysis was performed, and protein extracts underwent Western blot analysis. Representative immunoblot images illustrating the Nox4 isoforms (72 and 66 kDa) along with β-actin in C2C12 cells cultured in NG (A) or HG (B). Densitometry measurements of the NOX4 66 kDa isoform normalized to β-actin in C2C12 cells grown in NG (C) or HG (D) and the Nox4 72 kDa isoform normalized to β-actin in C2C12 cells cultured in NG (E) and HG (F). *P<0.05 vs veh control was considered significant.

Future Research and Impact:

Future investigations should delve into metformin's molecular mechanisms in regulating REDOX signaling in the differentiation of human skeletal muscle cells but not only using immortalised cell lines but primary cells. *In vitro* studies are essential in elucidating the molecular mechanisms of diabetes; however animal models of T2DM should be utilized to confirm *in vitro* results. Further research is required to understand what type of REDOX signalling pathways are regulated at different concentrations of metformin. In this investigation, we found that GKT was the most effective inhibitor to inhibit ROS in C2C12 myoblasts grown either in NG or HG. This result highlights the potential of targeting Nox1 and Nox4 as a therapeutic approach to treat patients with T2DM. Currently, another NOX1/NOX4 inhibitor, GKT137831, is under clinical trials for Type 1 and 2 diabetes (Thannickal et al. 2023). It would be interesting to examine in clinical trials the combinatorial effects of metformin and NOX1/NOX4 inhibition in treating T2DM. Furthermore, examining metformin's impact on broader aspects of diabetic skeletal muscle health, like mitochondrial function and autophagy, is utmost importance.

Importance and Beneficiaries:

This study enhances our understanding of the effects of metformin, and NOX inhibitors upon ROS production in skeletal muscle myoblasts grown in diabetic-mimic conditions. This research may identify NOXs as therapeutic targets to treat patients with T2DM who have mobility dysfunctions. Further research is required to evaluate the effects of varying concentrations of metformin to act as an anti-oxidant agent and whether combination treatment of metformin and NOX inhibitors can be used as a therapeutic strategy to treat T2DM.

Biochemical Society's Strategy:

This research aligns with the Biochemical Society's objective to apply biochemistry to address real-world health challenges, ultimately improving human health which is part of Goal 3 of the UN Sustainability goals.

Skills Acquired:

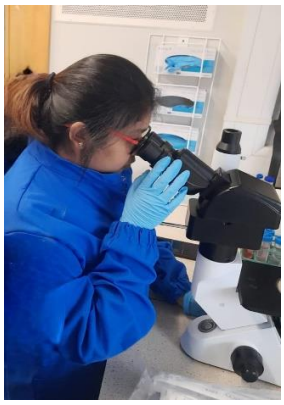
Practical Skills Acquired:
This studentship gave me the opportunity to learn several molecular biology techniques and bridging knowledge with hands-on expertise. Specifically, I have learned the following techniques: health and safety procedures, pipetting, cell culturing, ROS assays, and Western blotting. I have learned to perform experimental design, statistical/data analysis using Excel and GraphPad Prism 9 and critically analyze and interpret my results. Additionally, my verbal and written communication skills have improved significantly.

Transferable Skills Developed:

Through the project I developed strong time management skills, balancing tasks efficiently. Seeking guidance and integrating feedback became second nature, contributing to personal and project growth. Collaboration and communication skills thrived during my work, fostering effective teamwork. Organizational skills flourished, setting a sturdy foundation for my career.

My collaboration with Dr. Anagnostopoulou during this studentship has significantly deepened my commitment to pursuing a future career as a researcher in cardiovascular research. It provided me with the laboratory skills to use biochemical assays to elucidate the role of oxidative stress signaling in skeletal muscle myoblasts grown in diabetic-mimic conditions. This Biochemical society studentship has provided me the scientific foundation and employability skills required for me to progress my career development path to pursue a MSc then continue with a PhD degree and finally become an independent Biomedical Science researcher.

(A) (B)



(C) (D)

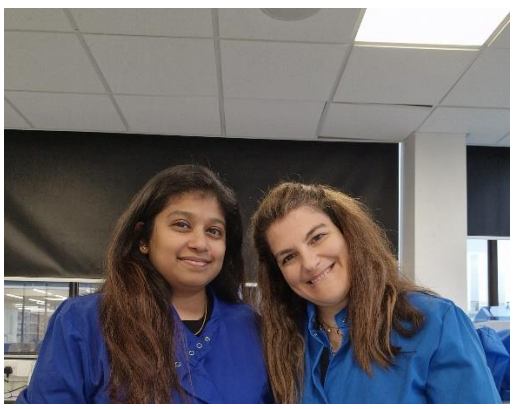


Figure 5: (A) Arshiya checking C2C12 cell growth under the microscope. (B) Arshiya pipetting media into a T25 flask containing C2C12 cells. (C) Arshiya Siddique with her Supervisor Dr Aikaterini Anagnostopoulou. (D) Arshiya running Western Blotting.

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