

Dose-Dependent Effect of Metformin Preconditioning on the Regenerative Potential of Human Umbilical Cord-Derived Mesenchymal Stem Cells: An In Vitro Experimental Study

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Abstract

Background

Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) show promise in regenerative medicine, but variable functional quality. This study evaluates the effects of metformin preconditioning across a wide concentration range on UC-MSC regenerative potential.

Methods

UC-MSCs (n = 10 donors) were isolated, characterized, and exposed to 0–1000 μ M metformin. Viability, colony-forming efficiency, proliferation kinetics (passages 3–6), and osteogenic/neurogenic differentiation were evaluated.

Results

Metformin modulated UC-MSC function in a dose-dependent manner. The 100 μ M dose significantly maximized cell viability (106.30%) and clonogenicity, while maintaining higher population doubling and shortening doubling time at passage 6 versus controls. Furthermore, 100 μ M metformin significantly upregulated osteogenic (OCN) and neurogenic (NSE) gene expression. Conversely, higher concentrations (1000 μ M) inhibited proliferation and differentiation.

Conclusions

Low-dose (100 μ M) metformin preconditioning enhances UC-MSC metabolic activity, proliferation, and differentiation potential. This optimization offers a pharmacological strategy to improve UC-MSC quality for therapeutic applications.

1. Introduction

Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) are widely studied for regenerative medicine. The umbilical cord is discarded after delivery, so harvesting is ethically uncomplicated and non-invasive. Cells from Wharton's jelly expand well in culture, show low senescence at the time of collection, and are easier to bank and manipulate than adult-derived MSCs [1]. These practical advantages have placed UC-MSCs among the most-used MSC sources in experimental and translational studies.

A consensus minimum criteria set defines MSCs through plastic adherence, expression of CD73, CD90, and CD105, absence of haematopoietic markers, and *in vitro* tri-lineage differentiation [2]. These criteria are useful but do not capture functional quality. MSC preparations vary in clonogenicity, growth rate,

senescence profile, differentiation responsiveness, and secretory activity. Source tissue, donor, and manufacturing differences are well documented [3,4]. Investigators are therefore moving towards functional readouts - colony-forming unit-fibroblast (CFU-F) assays, plating efficiency, population doubling, population doubling time, and lineage-specific gene expression - that better predict regenerative behaviour.

Ex vivo expansion stresses MSCs. Repeated trypsinisation, changes in oxygen tension, and prolonged passage erode clonogenicity and proliferation and may compromise differentiation. To counter this, pharmacological preconditioning has been explored. The aim is to expose cells to a defined, titratable agent under controlled conditions in order to improve subsequent functional performance.

Metformin, a biguanide used for over six decades in type 2 diabetes, has biological effects well beyond glucose lowering. It partially inhibits mitochondrial complex I, modulates the AMP-activated protein kinase (AMPK) pathway, influences mTOR signalling, and changes autophagy and redox balance [5]. Several of these pathways intersect with stem cell survival, proliferation, senescence, and lineage commitment, which is why metformin has attracted attention as an MSC modulator [6].

In UC-MSCs specifically, metformin's effects are concentration- and context-dependent. Low micromolar concentrations support proliferation; higher concentrations inhibit growth and shift differentiation balance [7]. Functional organic cation transporters mediate metformin uptake into human Wharton's jelly UC-MSCs and are required for the osteogenic response [8]. Other UC-MSC and perinatal MSC studies have linked metformin's osteogenic effect to PI3K/AKT/mTOR and AMPK-related pathways [9,10]. These signalling claims should be applied carefully to any given experimental model unless directly assayed.

The biphasic nature of metformin matters for preconditioning. A useful concentration must improve functional outcomes without compromising expansion. Dose-response screening is therefore the first step before committing to a single concentration for downstream experiments.

Metformin's influence on neurogenic differentiation-associated response in UC-MSCs is less well defined than its osteogenic effect. In adult neural stem cells, metformin promotes proliferation and neuronal differentiation through AMPK-dependent regulation [11]. UC-MSCs are not neural stem cells, so neuron-like morphology and neuron-specific marker expression in UC-MSCs should be reported as a neurogenic differentiation-associated response rather than mature neuronal conversion.

Despite a sizeable literature on metformin and MSCs, few studies in human UC-MSCs combine a dose-response screen with multiple functional endpoints in the same cell preparations. We therefore designed an integrated assessment of metabolic activity, clonogenicity, proliferative kinetics, osteogenic differentiation with OCN expression, and neurogenic differentiation-associated response with NSE expression. The objective was to identify a working preconditioning concentration that improves UC-MSC functional quality without compromising expansion.

2. Materials and Methods

2.1 Study Design and Setting

This was a comparative *in vitro* experimental study comparing untreated and metformin-preconditioned UC-MSCs from human Wharton's jelly. The work was carried out at the Advanced Research Centre of Biomedical Sciences, King Edward Medical University, Lahore, Pakistan, in the Stem Cell, DNA, RNA, and RT-PCR Laboratories under Biosafety Level-2 precautions from (Dec/2019 – Jan/2022) in accordance with the STROBE checklist for cohort studies mentioned in the supplementary appendix.

2.2 Ethical Approval and Consent

This was an *in vitro* study utilizing entirely human-derived cells; no animal subjects or animal models were involved. The study protocol was approved by the Institutional Ethics Review Committee of King Edward Medical University, Lahore (Approval No. 1030/RC/KEMU; dated 21 August 2019). Human umbilical cord tissue was collected after obtaining written informed consent from the mothers. Tissue collection followed routine clinical deliveries and did not alter obstetric management. No identifiable maternal or neonatal clinical information was retained.

2.3 Donor Eligibility and Sample Size

Umbilical cords were collected by non-probability convenience sampling from healthy term neonates delivered by Caesarean section when transport and immediate processing were feasible. Inclusion required APGAR ≥ 8 at birth, absence of congenital anomaly or major perinatal complication, and maternal consent. Cords were excluded for maternal gestational diabetes, hypertensive disorders of pregnancy, hepatitis B, hepatitis C, HIV infection, congenital fetal anomaly, or delayed processing. Ten independent donors ($n = 10$) contributed cells to each experimental arm. Sample size was based on availability, prior published *in vitro* MSC experiments using similar replicate numbers, and feasibility for the laboratory.

2.4 Cord Collection and Transport

A 5–10 cm segment of cord was collected aseptically and placed in a sterile 50 mL tube containing phosphate-buffered saline (PBS, pH 7.2) with penicillin 100 U/mL, streptomycin 100 $\mu\text{g/mL}$, and amphotericin B 0.25 $\mu\text{g/mL}$. The outer surface was wiped with 70% ethanol, washed with sterile PBS, and transported to the laboratory at 4–8°C within 1–2 h.

2.5 Isolation and Primary Culture of UC-MSCs

Cells were isolated by the explant method in a Class II biosafety cabinet. After PBS washing, the two arteries and the vein were dissected out, Wharton's jelly was separated, and the tissue was cut into 2–3 mm^3 explants. Thirty to forty explants were placed in each T-25 flask with sufficient spacing, with a minimal volume of medium for adherence. Flasks were left undisturbed for 48–72 h.

Culture medium was alpha minimum essential medium (α -MEM) supplemented with 10% MSC-qualified heat-inactivated fetal bovine serum, 1% L-glutamine/GlutaMAX, 1% penicillin-streptomycin, and 1% non-essential amino acids, filtered through a 0.22 μ m filter. Cultures were maintained at 37°C in 5% CO₂. Medium was changed every 2–3 days. Cells were examined daily for outgrowth, morphology, confluence, and contamination. All primary cultures were tested for mycoplasma before use in experimental assays and only mycoplasma-negative cultures were taken forward.

2.6 Passaging and Morphology

Cells were detached at 70–80% confluence using 0.25% trypsin-EDTA for 2–4 min at 37°C. Trypsin was neutralised with complete medium, and the suspension was centrifuged. Viable cells were counted by trypan blue exclusion using a haemocytometer and reseeded at 4,000–5,000 cells/cm². Experimental assays used early-passage cells (mainly P3-P5). Cells were inspected for plastic adherence, spindle-shaped fibroblast-like morphology, whorl-like arrangement, and cytoplasmic clarity.

2.7 Immunophenotypic Characterisation

Cells at P3–P5 were characterised by immunofluorescence following established MSC criteria [2]. Cells were seeded on sterile glass coverslips in 24-well plates and allowed to attach for 24–48 h. After PBS washing, cells were fixed with 4% paraformaldehyde for 15 min, permeabilised with 0.1% Triton X-100 in PBS for 5 min where required, and blocked with 1% bovine serum albumin in PBS for 30 min. Primary monoclonal antibodies targeted CD44, CD73, CD90, CD105, CD14, CD34, and CD45.

Tetramethylrhodamine-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rat secondary antibodies were applied in the dark. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted in anti-fade medium and examined on an inverted epifluorescence microscope. Mouse, rat, unstained, and secondary-only controls were used to assess background and non-specific staining.

2.8 Metformin Preparation and Preconditioning

Pharmaceutical-grade metformin hydrochloride was used. A 1 mM working stock was prepared under sterile conditions, filter-sterilised through a 0.22 μ m filter, aliquoted, and stored at 4°C. Working solutions were freshly diluted before each assay. For dose-response, cells were exposed to 50, 100, 200, 400, 800, and 1000 μ M metformin. Proliferation-related assays used 0, 100, 500, and 1000 μ M. Differentiation assays used 100 μ M as the preconditioning concentration based on the XTT result. Cells were seeded and allowed to attach for 24 h before metformin exposure. Controls received complete medium without metformin. Exposure duration was assay-specific: short-term exposure for XTT dose optimisation, exposure throughout colony formation for CFU-F, exposure during serial expansion for PD/PDT, and 48 h preconditioning before osteogenic or neurogenic induction.

2.9 XTT Cell Viability and Metabolic Activity

The XTT colorimetric assay was used to assess metabolic activity [12]. Cells were seeded in flat-bottom 96-well plates at 1×10^4 cells/well in 100 μ L medium and incubated for 24 h. The medium was then

replaced with metformin-containing or control medium. After exposure, freshly prepared XTT reaction mixture was added per the manufacturer's instructions and incubated 3–4 h at 37°C in the dark. Absorbance was read at 450 nm with a 640 nm reference correction. Blank wells (medium and XTT, no cells) were subtracted. Viability was expressed as a percentage of the untreated control.

2.10 CFU-F and Plating Efficiency

The CFU-F assay was used to assess clonogenic capacity [13]. Cells were seeded at low density in 6-well plates at 1000 cells/well, assigned to control or metformin-treated groups, and cultured for 10–14 days under standard conditions with regular medium renewal. Cultures were then fixed with 100% methanol and stained with 0.5% crystal violet. Colonies of ≥ 50 cells were counted. Plating efficiency (%) was calculated as (counted colonies / cells seeded) $\times$ 100.

2.11 Population Doubling and Population Doubling Time

PD and PDT were tracked during serial expansion. Cells were seeded at defined densities, harvested at 70–80% confluence, neutralised, centrifuged, and resuspended. Viable counts were averaged from three trypan blue counts. PD was calculated as $\log_2(N_{\text{h}} / N_{\text{s}})$, where N_{h} is the harvested viable count and N_{s} the seeded count. PDT was calculated as t / PD , where t is the duration of the interval in hours.

2.12 Osteogenic Differentiation

UC-MSCs at P3-P5 were seeded in 6-well plates at 5×10^3 viable cells/cm². After 24 h, cells were assigned to control or 100 μM metformin groups; the metformin group was preconditioned for 48 h before induction. Growth medium was then replaced with osteogenic induction medium (α -MEM with 10% FBS, 100 nM dexamethasone, 10 mM β -glycerophosphate, and 50 $\mu\text{g/mL}$ ascorbic acid; all from Sigma-Aldrich, St. Louis, MO, USA). Medium was changed every 3–4 days for 14 days. Outcomes included microscopic morphology, a semi-quantitative 1–5 osteogenic score, modified Von Kossa staining, and osteocalcin gene expression. At day 14, cultures were fixed with 4% paraformaldehyde and stained with silver nitrate under bright light, with sodium thiosulfate to remove unreacted silver. Mineralised areas were identified as dark deposits in the extracellular matrix and recorded by photomicrography.

2.13 Neurogenic Differentiation

UC-MSCs at P3-P5 were seeded in 6-well plates at $1.5\text{--}2.0 \times 10^4$ cells/cm². After 24 h, cells were assigned to control or 100 μM metformin groups; the metformin group was preconditioned for 48 h. Growth medium was replaced with chemical neurogenic induction medium (α -MEM with 10 ng/mL basic fibroblast growth factor for 24 h of pre-induction, followed by induction medium containing 2% dimethyl sulfoxide, 200 μM butylated hydroxyanisole, and 25 mM KCl in serum-free α -MEM), maintained at 37°C in 5% CO₂, with medium changed every 2–3 days for 7–10 days. Neurogenic response was assessed by morphology, haematoxylin and eosin staining, a semi-quantitative neurogenic morphology score, and NSE gene expression. Findings were interpreted as a neurogenic differentiation-associated response rather than mature neuronal conversion.

2.14 RNA Extraction, cDNA Synthesis, and RT-qPCR

Total RNA was extracted from designated differentiation wells using the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) per the manufacturer's instructions. RNA concentration and purity were checked before cDNA synthesis with the RevertAid cDNA synthesis kit (Thermo Scientific). RT-qPCR was performed on the Agilent AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) in 96-well format, with reporting guided by the MIQE quality principles [14]. Each 20 μ L reaction contained PCR master mix, forward and reverse primers, cDNA template, and nuclease-free water. Samples were run in technical triplicate. No-template controls monitored contamination. Cycling included initial activation followed by 40 cycles of denaturation, annealing, and extension. Melt-curve analysis was used to confirm amplification specificity. OCN served as the osteogenic marker, NSE as the neurogenic marker, and β -actin as the housekeeping reference. Relative expression was calculated by the $2^{(-\Delta\Delta Ct)}$ method [15].

2.15 Quality Control and Biosafety

Only early-passage UC-MSCs were used. Cultures were inspected daily and tested for mycoplasma before assays; only mycoplasma-negative cultures were used. Viability was assessed by trypan blue. Cultures with contamination, poor morphology, or technical artefacts were excluded from the affected analysis.

2.16 Statistical Analysis

Data are mean $\pm$ standard deviation (SD). Distribution was assessed by Shapiro-Wilk testing and homogeneity of variance by Levene's test before parametric analysis. For multiple-group comparisons, one-way ANOVA followed by Tukey HSD post-hoc tests was used. Two-group comparisons used independent *t*-tests. A two-sided *p*-value < 0.05 was considered significant. Analyses were performed in SPSS version 29 (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Morphology and Outgrowth

Explants attached within 48-72 h. Stromal cells migrated outward over the first 5-7 days. By days 12-14, cultures formed confluent monolayers of spindle-shaped, fibroblast-like cells arranged in characteristic whorl-like patterns, with clear cytoplasm and minimal granularity. Morphology was stable across passages 3-5 (Fig. 1A, 1B).

3.2 Immunophenotype

Immunofluorescence confirmed the mesenchymal phenotype. UC-MSCs expressed CD44, CD73, CD90, and CD105 and lacked CD14, CD34, and CD45. Mouse and rat control preparations showed minimal non-

specific staining. These findings confirmed isolation of UC-MSCs suitable for the planned functional assays (Fig. 1C-1I).

3.3 Dose-Dependent Effect of Metformin on Metabolic Activity

XTT readings showed a concentration-dependent effect of metformin. Untreated cells set the baseline at $100.00 \pm 0.00\%$. Mean viability rose to $102.18 \pm 0.45\%$ at 50 μM and reached $106.30 \pm 0.89\%$ at 100 μM . Values were $100.74 \pm 0.57\%$ at 200 μM and $102.93 \pm 0.56\%$ at 400 μM , indicating a non-monotonic profile in the mid-range. Viability then declined to $99.55 \pm 1.03\%$ at 800 μM and $95.34 \pm 0.94\%$ at 1000 μM . One-way ANOVA on percentage viability showed a significant overall effect of concentration ($F = 222.58, p < 0.0001; n = 10$ donors per concentration). The 100 μM concentration was carried forward for osteogenic and neurogenic experiments (Table 1, Fig. 2).

Interpretation first, then table: the XTT data describe a beneficial concentration around 100 μM and an inhibitory trend at the highest concentrations.

Table 1. XTT metabolic viability of UC-MSCs across metformin concentrations

Metformin (μM)	Mean viability (%)	SD	n
0	100.00	0.00	10
50	102.18	0.45	10
100	106.30	0.89	10
200	100.74	0.57	10
400	102.93	0.56	10
800	99.55	1.03	10
1000	95.34	0.94	10

Abbreviations: SD, standard deviation; UC-MSC, umbilical cord-derived mesenchymal stem cell. Viability is expressed relative to untreated controls after background subtraction. One-way ANOVA: $F = 222.58, p < 0.0001$.

3.4 Effect of Metformin on Clonogenic Capacity and Plating Efficiency

Clonogenicity followed the same broad pattern as XTT. Untreated cultures produced 52.6 colonies per dish ($5.26 \pm 0.77\%$ plating efficiency). The 100 μM group produced 65.7 colonies per dish and the highest plating efficiency ($6.57 \pm 0.59\%$). The 500 μM group produced 45.9 colonies per dish ($4.59 \pm 1.06\%$), and the 1000 μM group produced 33.8 colonies per dish ($3.38 \pm 0.91\%$). One-way ANOVA on CFU-F percentage showed a significant effect of concentration ($F(3,36) = 24.74, p < 0.001$). Tukey HSD post-hoc analysis showed that 100 μM increased plating efficiency over control ($p < 0.01$); 500 μM and

1000 µM were both lower than 100 µM ($p < 0.001$), and 1000 µM showed the greatest suppression (Table 2, Fig. 3).

Interpretation first, then table:

Table 2. CFU-F and plating efficiency across metformin concentrations

Metformin (µM)	Mean colonies / dish	Plating efficiency (%) ± SD	N	Tukey group
0	52.6	5.26 ± 0.77	10	B
100	65.7	6.57 ± 0.59	10	A
500	45.9	4.59 ± 1.06	10	B/C
1000	33.8	3.38 ± 0.91	10	C

Abbreviations: CFU-F, colony-forming unit-fibroblast; SD, standard deviation. ANOVA: $F(3,36) = 24.74$, $p < 0.001$. Different Tukey groups indicate statistically different post-hoc groupings.

3.5 Effect of Metformin on Population Doubling and Population Doubling Time

Serial expansion from P3 to P6 showed that metformin altered proliferative kinetics in a concentration-dependent fashion. In control cultures, PD declined from 1.31 ± 0.06 at P3 to 0.95 ± 0.04 at P6, with PDT increasing from 55.03 ± 2.39 h to 86.51 ± 5.22 h.

The 100 µM group maintained the best PD profile across all passages: 1.63 ± 0.07 (P3), 1.53 ± 0.08 (P4), 1.40 ± 0.04 (P5), and 1.30 ± 0.06 (P6). PDT was correspondingly shorter: 44.07 ± 2.19 h, 48.65 ± 3.20 h, 55.39 ± 2.91 h, and 62.05 ± 3.40 h, respectively.

Higher concentrations were inhibitory. In the 500 µM group, PD fell from 1.22 ± 0.03 to 0.75 ± 0.03 across P3–P6, and PDT rose from 56.06 ± 4.01 h to 111.56 ± 6.65 h. The 1000 µM group declined most: PD from 1.01 ± 0.04 to 0.52 ± 0.02 and PDT from 71.26 ± 2.65 h to 157.63 ± 10.89 h. One-way ANOVA confirmed a significant effect of concentration on PD and PDT at each passage ($p < 0.001$) (Table 3, Fig. 4).

Interpretation first, then table:

Table 3. PD and PDT across passages by metformin concentration

Passage	Metformin (μM)	PD (mean $\pm$ SD)	PDT, h (mean $\pm$ SD)
P3	0	1.31 $\pm$ 0.06	55.03 $\pm$ 2.39
P3	100	1.63 $\pm$ 0.07	44.07 $\pm$ 2.19
P3	500	1.22 $\pm$ 0.03	56.06 $\pm$ 4.01
P3	1000	1.01 $\pm$ 0.04	71.26 $\pm$ 2.65
P4	0	1.23 $\pm$ 0.08	60.17 $\pm$ 6.12
P4	100	1.53 $\pm$ 0.08	48.65 $\pm$ 3.20
P4	500	1.09 $\pm$ 0.13	69.25 $\pm$ 9.99
P4	1000	0.87 $\pm$ 0.10	88.21 $\pm$ 12.10
P5	0	1.07 $\pm$ 0.05	72.98 $\pm$ 3.90
P5	100	1.40 $\pm$ 0.04	55.39 $\pm$ 2.91
P5	500	0.89 $\pm$ 0.03	89.88 $\pm$ 6.25
P5	1000	0.63 $\pm$ 0.02	125.71 $\pm$ 7.73
P6	0	0.95 $\pm$ 0.04	86.51 $\pm$ 5.22
P6	100	1.30 $\pm$ 0.06	62.05 $\pm$ 3.40
P6	500	0.75 $\pm$ 0.03	111.56 $\pm$ 6.65
P6	1000	0.52 $\pm$ 0.02	157.63 $\pm$ 10.89

Abbreviations: PD, population doubling; PDT, population doubling time; SD, standard deviation. $n = 10$ donors per group at each passage. ANOVA $p < 0.001$ at each passage.

3.6 Osteogenic Differentiation After 100 μM Metformin Preconditioning

Both groups showed osteoblast-like morphological change and matrix deposition during induction, but the 100 μM metformin-preconditioned group was clearly more advanced. Cell density was higher, matrix deposition was more apparent, and modified Von Kossa staining showed denser mineralisation. The semi-quantitative osteogenic morphology score rose from 3.25 ± 0.50 in controls to 3.63 ± 0.52 in the metformin group ($p < 0.05$). Relative OCN expression rose from 1.00 ± 0.17 to 1.85 ± 0.21 ($p < 0.05$). These findings indicate enhanced osteogenic differentiation after 100 μM metformin preconditioning (Table 4, Fig. 5).

3.7 Neurogenic Differentiation-Associated Response

Neurogenic induction produced neuron-like morphological changes in both groups. Control cultures showed cytoplasmic retraction, compact cell bodies, and limited bipolar or multipolar processes. The

100 μ M metformin-preconditioned group developed more defined cell bodies, longer neurite-like processes, and more advanced bipolar or multipolar morphology. Haematoxylin and eosin staining confirmed these features. The semi-quantitative neurogenic morphology score increased from 1.75 ± 0.46 to 2.88 ± 0.64 ($p = 0.043$). Relative NSE expression increased from 1.00 ± 0.12 to 2.41 ± 0.53 ($p = 0.029$). Findings are interpreted as a neurogenic differentiation-associated response rather than mature neuronal conversion (Table 4, Fig. 6).

Table 4. Osteogenic and neurogenic differentiation-associated outcomes

Assay	Outcome	Control	100 μ M metformin	p-value
Osteogenic	Morphology score	3.25 ± 0.50	3.63 ± 0.52	< 0.05
Osteogenic	Relative OCN	1.00 ± 0.17	1.85 ± 0.21	< 0.05
Neurogenic	Morphology score	1.75 ± 0.46	2.88 ± 0.64	0.043
Neurogenic	Relative NSE	1.00 ± 0.12	2.41 ± 0.53	0.029

Values are mean $\pm$ SD; $n = 10$ donors per group. Abbreviations: NSE, neuron-specific enolase; OCN, osteocalcin; SD, standard deviation. Statistical tests: independent t -test for each comparison.

3.8 Summary of Findings

Across all functional readouts, 100 μ M metformin was the most favourable concentration. It produced the highest XTT viability, the highest CFU-F/plating efficiency, the best-maintained PD/PDT across serial expansion, and the strongest osteogenic and neurogenic-associated responses. Higher concentrations, particularly 1000 μ M, were inhibitory. Lower concentrations (50 μ M) were less effective than 100 μ M but did not reduce viability.

4. Discussion

The main finding of this study is a clear concentration-dependent effect of metformin on UC-MSC regenerative potential, with 100 μ M as the most favourable preconditioning concentration. At 100 μ M, XTT viability rose from $100.00 \pm 0.00\%$ to $106.30 \pm 0.89\%$, plating efficiency from $5.26 \pm 0.77\%$ to $6.57 \pm 0.59\%$, P6 PD from 0.95 ± 0.04 to 1.30 ± 0.06 , and P6 PDT fell from 86.51 ± 5.22 h to 62.05 ± 3.40 h. The same concentration enhanced osteogenic and neurogenic-associated responses, with OCN rising from 1.00 ± 0.17 to 1.85 ± 0.21 and NSE from 1.00 ± 0.12 to 2.41 ± 0.53 . At 1000 μ M, viability, clonogenicity, and serial expansion were all reduced. The pattern supports the idea of a useful biological window for metformin preconditioning rather than a uniformly positive effect.

The biphasic response is consistent with previous UC-MSC research. In the study conducted by Lei et al., metformin at a concentration of 0-500 μ M stimulated the proliferation of UC-MSC, but 1000 μ M inhibited it; they also found that metformin affected the osteogenic-adipogenic balance [7]. Our highest response

with XTT was at 100 μ M, and at 1000 μ M the viability was $95.34 \pm 0.94\%$, which is similar to that report. Bajetto et al. applied much higher concentrations (0.1-50 mM) for 48 h in human UC-MSCs and observed a dose-dependent decrease in cell growth, which was statistically significant at 3 mM and maximal at 50 mM, as well as reduced colony formation [16]. The exposures in that study are much higher than ours, but both studies suggest the same concept: that too much metformin exposure results in impaired UC-MSC growth and clonogenic capacity.

Clonogenicity is informative because it reflects the proportion of cells retaining attachment, survival, and proliferative capacity. In our experiments, 100 μ M metformin raised mean colony count from 52.6 to 65.7 per dish and plating efficiency from $5.26 \pm 0.77\%$ to $6.57 \pm 0.59\%$; 500 μ M and 1000 μ M reduced plating efficiency to $4.59 \pm 1.06\%$ and $3.38 \pm 0.91\%$, respectively. Acar et al. reported that long-term metformin exposure (6 weeks of therapeutic-range treatment) reduced replicative senescence and cell death in human adipose MSCs and modulated transcription factors linked to ROS scavenging [17]. Their model is different from ours in cell source, concentration, and duration, but the broader message - that metformin can preserve growth-related MSC function under selected culture conditions - is consistent with what we observed.

The effect of metformin on proliferation is not consistently in the same direction. Chinnapaka et al. found that metformin enhanced the stemness of human adipose-derived stem cells, and decreased the proliferation rate and adipogenic differentiation, and inhibited mTOR and ERK activity, and promoted autophagy [18]. That is not a contradiction of our findings, but rather an indication that enhanced stemness and enhanced proliferation do not necessarily lead to the same result. In our model, 100 μ M metformin enhanced metabolic activity and proliferative kinetics, but higher concentrations inhibited growth. The effect should therefore always be interpreted with regard to cell source, dose, duration, passage and endpoint.

The improvement in PD and PDT at 100 μ M is biologically significant as MSCs can be viable without losing functional expansion. Kim et al. demonstrated that MSCs derived from chronic kidney disease donors exhibited decreased proliferation, premature senescence, and increased DNA damage compared with control MSCs, and that preconditioning with metformin prevented these defects and enhanced the paracrine effects of MSCs on renal inflammation and fibrosis in their mouse model [19]. Le Pelletier et al. also found that metformin decreased oxidative stress, decreased mitochondrial dysfunction, decreased stress-induced senescence and enhanced adipocyte function in aged-donor adipose stromal cells [20]. These reports give us a mechanistic context on the PD/PDT pattern in our UC-MSCs.

The osteogenic results are among the strongest. At 100 μ M, the osteogenic morphology score and relative OCN expression both increased significantly. The result agrees with several UC-MSC and perinatal MSC studies. Al Jofi et al. showed that metformin at a clinically relevant concentration activated AMPK in human Wharton's jelly UC-MSCs, enhanced mineralised nodule formation, and increased RUNX2 expression, and that pharmacological or genetic inhibition of organic cation transporters reduced uptake and blunted the osteogenic response [8]. Manochantr et al. reported that

10–160 μM metformin increased viability of placenta-derived MSCs, while 80–320 μM enhanced ALP activity, matrix mineralisation, and osteogenic gene expression. At 320 μM on day 28, RUNX2, OSX, OCN, and COL1 rose approximately 5.7-, 4.5-, 2.8-, and 3.6-fold, respectively [10]. Our roughly 1.85-fold increase in OCN at 100 μM in UC-MSCs is lower than those values, which is plausible given differences in cell source, induction duration, donor variability, and endpoint timing.

Several studies in other MSC sources support the same direction of effect. Fan *et al.* reported that metformin-modulated macrophage-conditioned medium increased ALP, RUNX2, OCN, and COL1 expression in BMSCs and enhanced ALP activity and mineralised nodule formation [21]. Zhang *et al.* showed that 40 mM high glucose impaired proliferation, migration, and osteogenic differentiation of rat adipose-derived stem cells, while 0.1 mM metformin reversed those changes through autophagy activation and mTOR/GSK3 β modulation [22]. Dong *et al.* reported that metformin enhanced osteogenic activity of rat BMSCs under diabetic oxidative stress conditions [23]. Older mechanistic work also fits. Wang *et al.* reported that metformin induced osteoblastic differentiation and mineralisation in human iPSC-derived MSCs at clinically relevant concentrations [24]. Ma *et al.* found that 100 μM metformin significantly promoted osteogenic differentiation of human BMSCs through GSK3 β , Wnt/ β -catenin, and AMPK signalling [25]. Marycz *et al.* reported reduced ROS, enhanced osteogenic properties of adipose-derived MSCs *in vitro*, and increased bone density *in vivo* [26]. Gao *et al.* showed that 100 μM metformin for 21 days raised osteoblast-marker mRNA and lowered adipocyte-marker mRNA in rat marrow MSCs [27]. Our 100 μM finding aligns particularly closely with Ma *et al.* and Gao *et al.*

The neurogenic findings need a more cautious interpretation. We saw an increase in neurogenic morphology score (1.75 ± 0.46 to 2.88 ± 0.64) and in NSE expression (1.00 ± 0.12 to 2.41 ± 0.53) at 100 μM . Morphology and NSE alone cannot prove mature neuronal conversion or electrophysiological function. Cai *et al.* used a chitosan hydrogel supplemented with metformin and reported promotion of neuron-like differentiation of gingival MSCs, with proteomic upregulation of neural regeneration-related proteins, but explicitly noted that the cells did not become mature neurons [28]. The same caution applies here: our findings should be reported as a neurogenic differentiation-associated response.

Neural precursor work provides biological plausibility but does not transfer directly. Fatt *et al.* reported that metformin promoted proliferation, self-renewal, and differentiation of adult neural precursor cells; proliferation/self-renewal involved TAp73, while neuronal differentiation involved the AMPK-aPKC-CBP pathway [29]. Wang *et al.* earlier showed that metformin activated an atypical PKC-CBP pathway to promote neurogenesis and improve spatial memory formation in experimental systems [30]. Xing *et al.* recently reported that 1 μM metformin increased EdU-positive neural stem cells from $31.92 \pm 1.95\%$ to $43.80 \pm 1.55\%$ and Tuj-1-positive cells from $7.22 \pm 0.80\%$ to $13.58 \pm 0.99\%$ *in vitro*; in their spinal cord injury model, metformin raised Nestin-positive cells from $52.50 \pm 6.40/\text{mm}^2$ to $147.30 \pm 19.88/\text{mm}^2$ and the proportion of Tuj-1-positive cells among Nestin-positive cells from $15.00 \pm 1.08\%$ to $43.00 \pm 2.35\%$ [11]. These observations support a role for metformin in neural-lineage systems, but UC-MSCs and neural stem cells are biologically distinct. Our NSE increase should therefore be read as supportive of neurogenic responsiveness, not as proof of mature neuronal identity.

This caution is supported by MSC neurogenic-differentiation reviews. Neirinckx *et al.* emphasised that adult MSCs may express neural markers and adopt neuron-like morphology, but these findings cannot establish true functional neuronal differentiation without stronger phenotypic and electrophysiological evidence [31]. Our results are still meaningful because both morphology score and NSE expression increased, but the interpretation must remain bounded.

A plausible explanation for the observed pattern is moderate metabolic modulation. Metformin partially inhibits mitochondrial complex I, alters the AMP/ATP balance, and modulates AMPK-related pathways [5]. AMPK acts as a central metabolic sensor and coordinates the response to energetic stress and mitochondrial homeostasis [32]. AMPK and mTOR are also linked through autophagy regulation, including ULK1 phosphorylation [33]. Because mTOR regulates metabolism, autophagy, survival, proliferation, and migration, excessive inhibition may suppress growth, while moderate modulation may improve adaptive stress responses [34]. This framework explains why 100 μ M metformin was favourable in our UC-MSCs, while 500 μ M and 1000 μ M were not.

Several strengths support the conclusions. First, cells were characterised morphologically and immunophenotypically before functional testing, and only mycoplasma-negative early-passage cultures were used. Second, the study did not rely on a single viability assay; it integrated XTT metabolic activity, CFU-F/plating efficiency, PD, PDT, osteogenic differentiation, neurogenic differentiation-associated morphology, and lineage-specific gene expression. Third, a dose-response design produced a clear concentration-dependent pattern, which strengthens the central conclusion that metformin requires careful titration before use as a preconditioning agent.

There are also limitations. First, this was an *in vitro* study, so the findings cannot be transferred directly to *in vivo* therapeutic efficacy. Second, mechanistic assays were not performed. AMPK phosphorylation, mTOR activity, mitochondrial respiration, mitochondrial membrane potential, ROS levels, senescence markers, and autophagy markers should be added in future work. Third, the neurogenic readout used morphology, H&E staining, semi-quantitative scoring, and NSE expression only. Future work should add β III-tubulin, MAP2, NeuN, synaptophysin, GFAP, protein-level validation, and electrophysiological testing. Fourth, secretome and extracellular vesicle endpoints were not evaluated; MSC-mediated regeneration is partly mediated through paracrine pathways. Fifth, immunophenotyping used immunofluorescence rather than flow cytometry, so positivity percentages against the ISCT minimum-criteria thresholds were not quantified.

Secretome and EV studies are particularly relevant for follow-up. MSC-derived EVs are increasingly seen as mediators of immunomodulation and regeneration, and may represent a next-generation cell-free platform [35]. EV cargo varies with MSC tissue source and culture conditions [36,37]. If metformin improves UC-MSC metabolic fitness, it may also alter secretome or EV cargo, but this cannot be assumed without direct testing. Future studies should include cytokine profiling, EV isolation and characterisation, and compliance with MISEV reporting [38,39].

Finally, the inhibitory effect at higher concentrations should be emphasised. The 1000 μM exposure reduced plating efficiency to $3.38 \pm 0.91\%$, reduced P6 PD to 0.52 ± 0.02 , and increased P6 PDT to 157.63 ± 10.89 h. High-dose metformin was therefore not suitable for UC-MSC preconditioning under the tested conditions. This matters for translation, because a preconditioning agent should improve functional quality without compromising expansion.

Taken together, metformin preconditioning altered UC-MSC regenerative potential in a concentration-dependent manner. The 100 μM concentration was the most favourable under our conditions, improving metabolic viability, clonogenicity, serial expansion, osteogenic differentiation, and neurogenic differentiation-associated response. Higher concentrations, particularly 1000 μM , impaired clonogenicity and proliferative kinetics. The findings support low-dose metformin as a candidate preconditioning agent for selected regenerative attributes of UC-MSCs, while highlighting the need for mechanistic, secretome-based, and translational follow-up.

5. Conclusions

Metformin preconditioning influenced UC-MSC regenerative potential in a concentration-dependent fashion. Among the concentrations tested, 100 μM showed the most favourable profile, improving metabolic viability, clonogenicity, plating efficiency, PD, PDT, osteogenic differentiation, OCN expression, neurogenic differentiation-associated morphology, and NSE expression. Higher concentrations, particularly 1000 μM , reduced clonogenicity and impaired proliferative kinetics. These results support low-dose metformin as a candidate preconditioning strategy for selected regenerative attributes of UC-MSCs under defined *in vitro* conditions. Mechanistic, secretome-based, and translational follow-up are required before therapeutic application.

Declarations

Ethics Approval and Consent to Participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Ethical approval from an animal review committee was not required because no animals were used for research in this study. All human experimental protocols were approved by the Institutional Ethics Review Committee of King Edward Medical University, Lahore (Approval No. 1030/RC/KEMU; dated 21 August 2019). Human umbilical cord tissue was collected following routine delivery after written informed consent was obtained from the mothers. The study was conducted in accordance with institutional guidelines and did not alter routine obstetric management.

Consent for Publication

Not applicable. The manuscript does not contain identifiable individual-level personal data or clinical images.

Availability of Data and Materials

Data supporting the findings of this study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

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Author Contributions

MJM and MMB designed the study; MJM, MMB, NY, and MS designed the methodology; MJM and AJG performed the investigation and laboratory work; MJM performed the formal analysis and data curation; MJM drafted the manuscript; MJM, MMB, NY, MS, and AJG reviewed and edited the manuscript; MMB, NY, and MS supervised the project; MMB administered the project. FM reviewed the manuscript and submitted the full length manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

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Figures

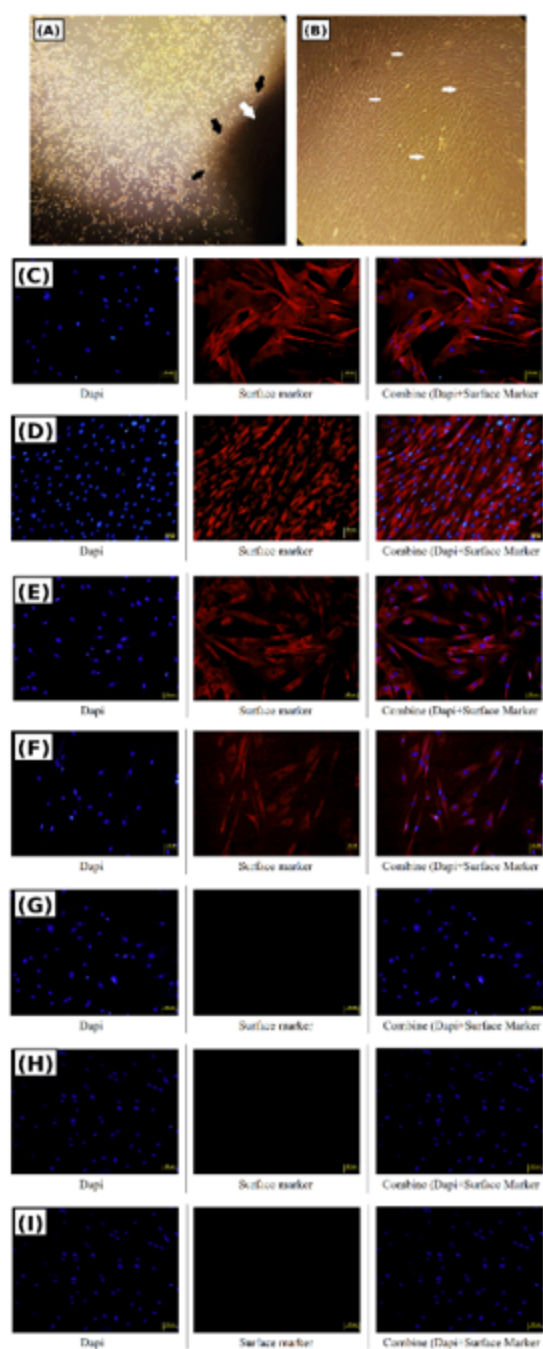


Figure 1

Morphology and immunophenotypic characterisation of UC-MSCs. (A) Early outgrowth of rounded stromal cells adjacent to the umbilical cord explant edge on day 2. (B) Confluent spindle-shaped fibroblast-like UC-MSC monolayer at days 12–14 showing characteristic whorl-like arrangement. (C–F) Immunofluorescence staining showing positive expression of CD44 (C), CD90 (D), CD105 (E), and CD73 (F). (G–I) Immunofluorescence staining showing absence of CD34 (G), CD45 (H), and CD14 (I). Each

immunofluorescence panel shows, from left to right, DAPI nuclear staining (blue), the surface marker channel (red), and the merged image. Scale bars: 150 μm .

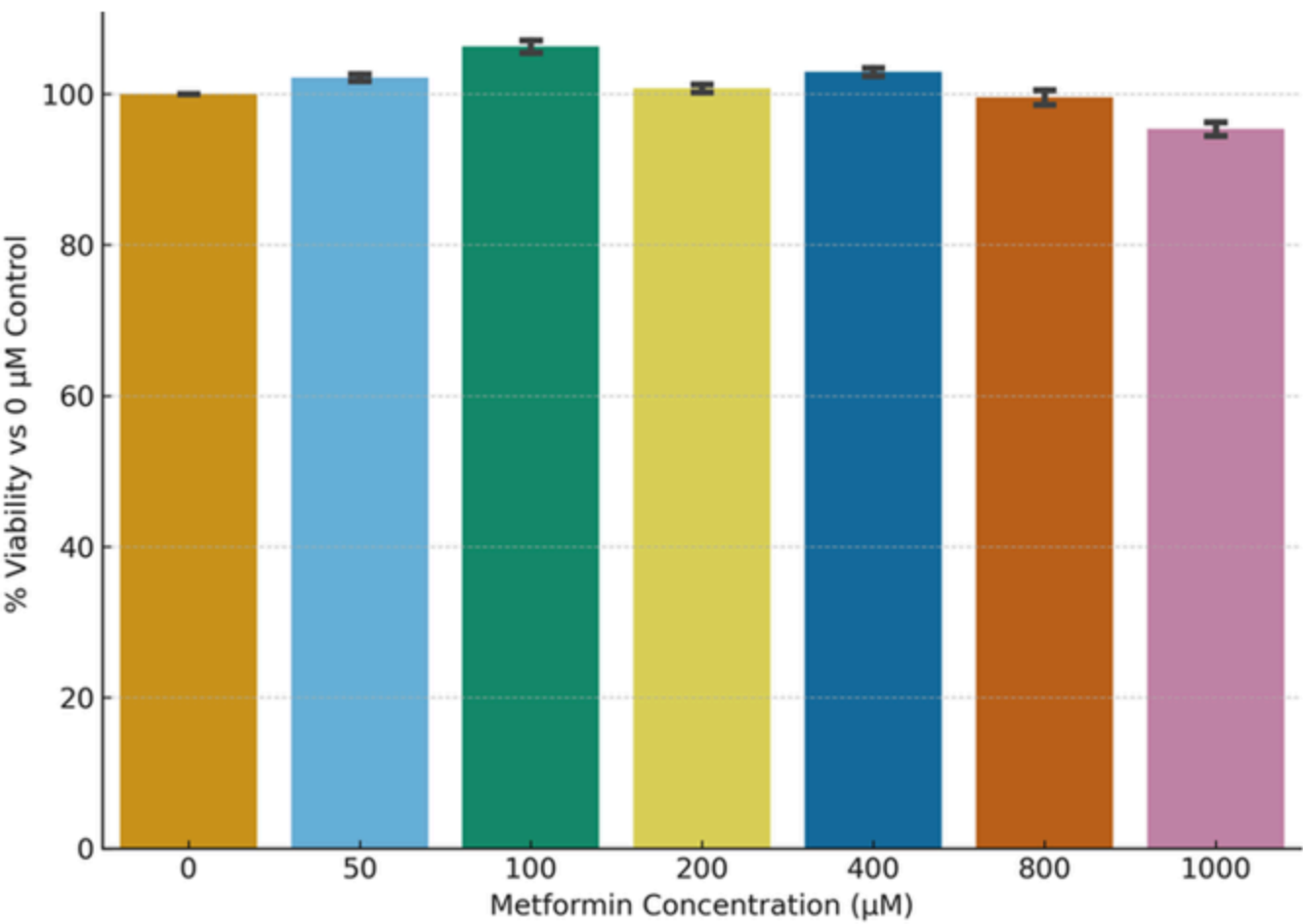


Figure 2

XTT viability of UC-MSCs after metformin preconditioning. Bars represent mean viability (%) relative to untreated control; error bars represent SD; $n = 10$ donors per concentration. One-way ANOVA showed a significant concentration-dependent effect ($F = 222.58$, $p < 0.0001$).

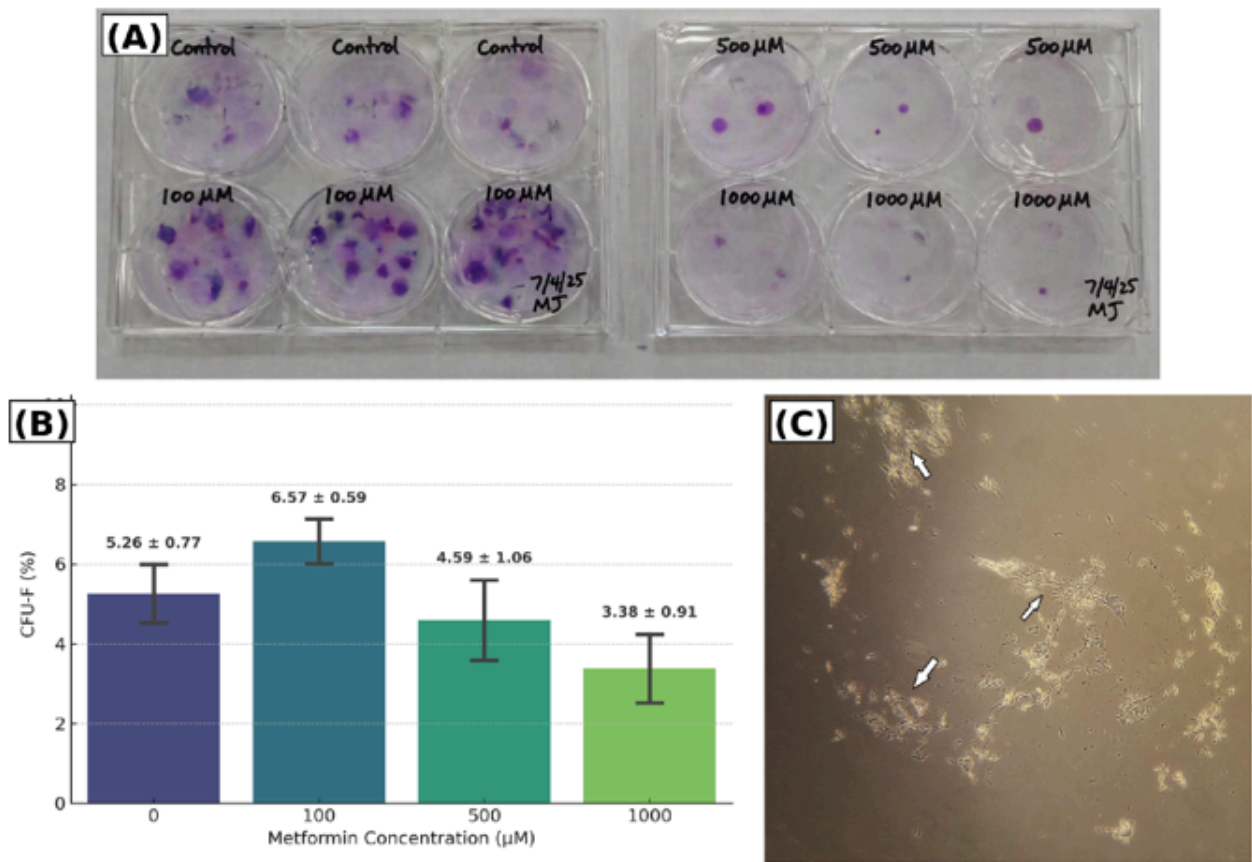


Figure 3

CFU-F and plating efficiency of UC-MSCs after metformin preconditioning. (A) Representative crystal-violet-stained CFU-F assay plates showing colony formation in control, 100, 500, and 1000 μM metformin groups. (B) CFU-F percentage across control and metformin-treated groups ($n = 10$ donors per group). (C) Representative phase-contrast image of dying colonies in the 1000 μM metformin group. ANOVA $F(3,36) = 24.74$, $p < 0.001$.

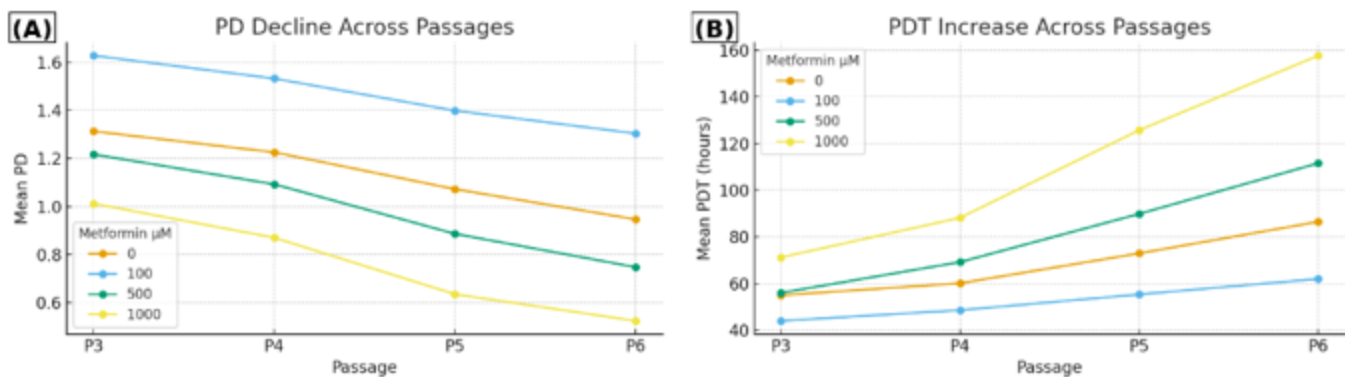


Figure 4

Population doubling (PD) and population doubling time (PDT) of UC-MSCs during serial expansion. (A) PD decline from P3 to P6 across control, 100, 500, and 1000 μ M metformin groups. (B) PDT increase from P3 to P6 across the same groups. $n = 10$ donors per group at each passage. One-way ANOVA showed significant concentration-dependent differences at each passage ($p < 0.001$).

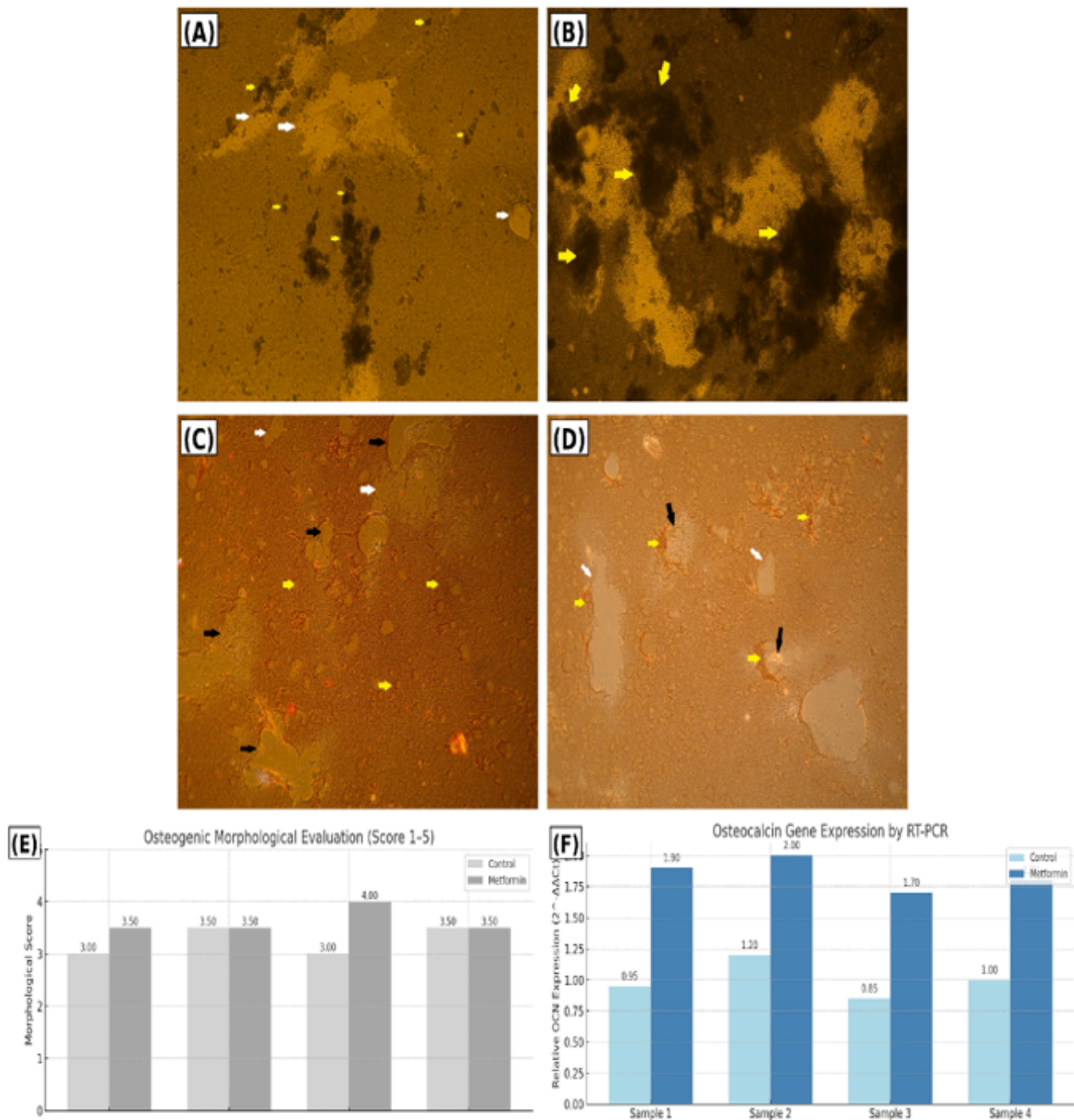


Figure 5

Osteogenic differentiation of UC-MSCs after 100 μ M metformin preconditioning at day 14. (A) Osteogenic morphology in the control group showing mineralisation foci (yellow arrows). (B) Osteogenic morphology in the 100 μ M metformin-preconditioned group with dense mineralisation foci (yellow arrows). (C) Modified Von Kossa staining in the control group with mineral-associated extracellular

matrix deposits (yellow arrows) and osteogenic foci (black arrows). (D) Modified Von Kossa staining in the 100 μ M metformin-preconditioned group. (E) Semi-quantitative osteogenic morphology score across donors. (F) Relative osteocalcin (OCN) gene expression by RT-qPCR; light bars represent control, dark bars represent 100 μ M metformin group. Independent *t*-test; $p < 0.05$ for both score and OCN.

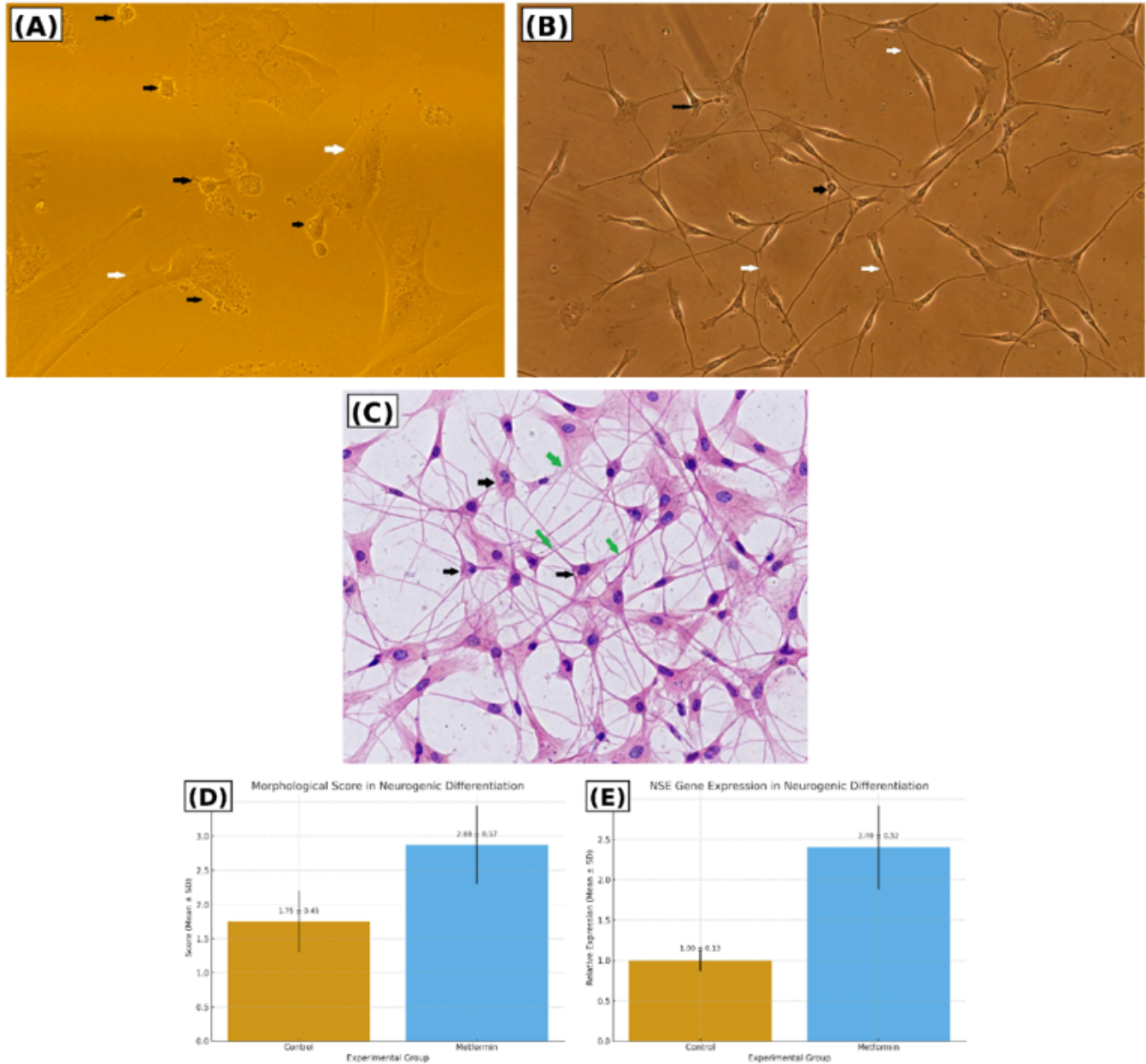


Figure 6

Neurogenic differentiation-associated response of UC-MSCs after 100 μ M metformin preconditioning. (A) Initial neurogenic response at day 7 in the control group, showing rounded perikarya (black arrows) and early cytoplasmic processes (white arrows). (B) Advanced neuron-like morphology in the 100 μ M metformin-preconditioned group at day 14 with extended neurite-like processes (white arrows) and recognisable neuron-like cell bodies (black arrows). (C) Haematoxylin and eosin-stained

photomicrograph of differentiated neuron-like cells in the 100 μ M metformin group showing prominent perikarya (black arrows) and neurite networks (green arrows). (D) Semi-quantitative neurogenic morphology score; control 1.75 ± 0.46 versus 100 μ M metformin 2.88 ± 0.64 ; $p = 0.043$. (E) Relative neuron-specific enolase (NSE) gene expression by RT-qPCR; control 1.00 ± 0.12 versus 100 μ M metformin 2.41 ± 0.53 ; $p = 0.029$. Independent t -tests; $n = 10$ donors per group. Findings should be interpreted as a neurogenic differentiation-associated response, not as mature neuronal conversion.