Human trabecular meshwork stem cell derived exosomes enhanced wound healing and antioxidant property: an attempt towards a cell-free therapy for glaucoma

Introduction

Glaucoma is an optic neuropathy, which is the second leading cause of irreversible blindness worldwide. It is estimated that about 111.8 million people will be affected by glaucoma by 2040 (Allison *et al.*, 2020). Trabecular meshwork (TM), a tiny porous tissue located in the iridocorneal angle of the eye that is responsible for intraocular pressure (IOP) homeostasis. In glaucomatous condition, the TM cellularity is reduced, altered extracellular matrix (ECM) leading to changes including increased stiffness, increased IOP thereby optic nerve damage and eventually blindness if untreated (Yun *et al.*, 2016). Previous report from our lab demonstrated that, in glaucomatous condition the adult tissue residence stem cells of TM (TMSC) located in the insert region are significantly lost compared to age-matched control (Sundaresan *et al.*, 2021).

To compensate these unsolicited glaucomatous alterations, stem cell transplantations using mesenchymal stem cells (MSC) such as BMSCs and ADSCs have been reported (Manuguerra-Gagne *et al.*, 2013, Zhou *et al.*, 2020). However, among the stem cells, adult tissue resident stem cells could be a better source as they naturally exist to maintain tissue homeostasis. Transplantation of TMSC in human organ culture of anterior segment (HOCAS-Iswarya *et al.*, ISSCR 2021) and animal models of glaucoma (Xiong *et al.*, 2021,) confirmed the therapeutic efficacy of TMSCs. However, the main obstacle in cell-based therapy includes the maintenance of stem cells in culture until transplantation emphasizing the need for better alternate.

Exosomes are nanovesicles (30-200nm) shed out of cells for cell-to-cell communications thereby mediating many cellular processes (Hamzah *et al.*, 2021). The exosomes mimic the nature of the parental cell in terms of contents including specific proteins, nucleic acids and lipids thereby might possess the functional property of the cell (Zhang *et al.*, 2019). The other advantages of exosomes are these tiny vesicles can cross even blood brain barrier, can be specifically targeted, non-immunogenic, easy to handle and store. Hence, the current research strategies are being focused on utilizing the efficacy of exosomes as an alternative to cell based therapies. Currently there are more than 250 clinical trials on exosomes which emphasise their significance as an alternative medicine (https://clinicaltrials.gov).

Stamer *et al.*, (2011) isolated exosomes from TM cells and illustrated their protein profile. Lerner *et al.*, (2019) demonstrated the significance of exosome-mediated communication between non-pigmented ciliary epithelial cells (NPCE) and TM. Till date, there are no reports available on exosomes from TMSC and their role in tissue homeostasis. The current study aimed to evaluate the efficacy of TM / TMSC exosomes and to identify the cargo which will form the base for cell-free therapy for glaucoma in future.

Objectives

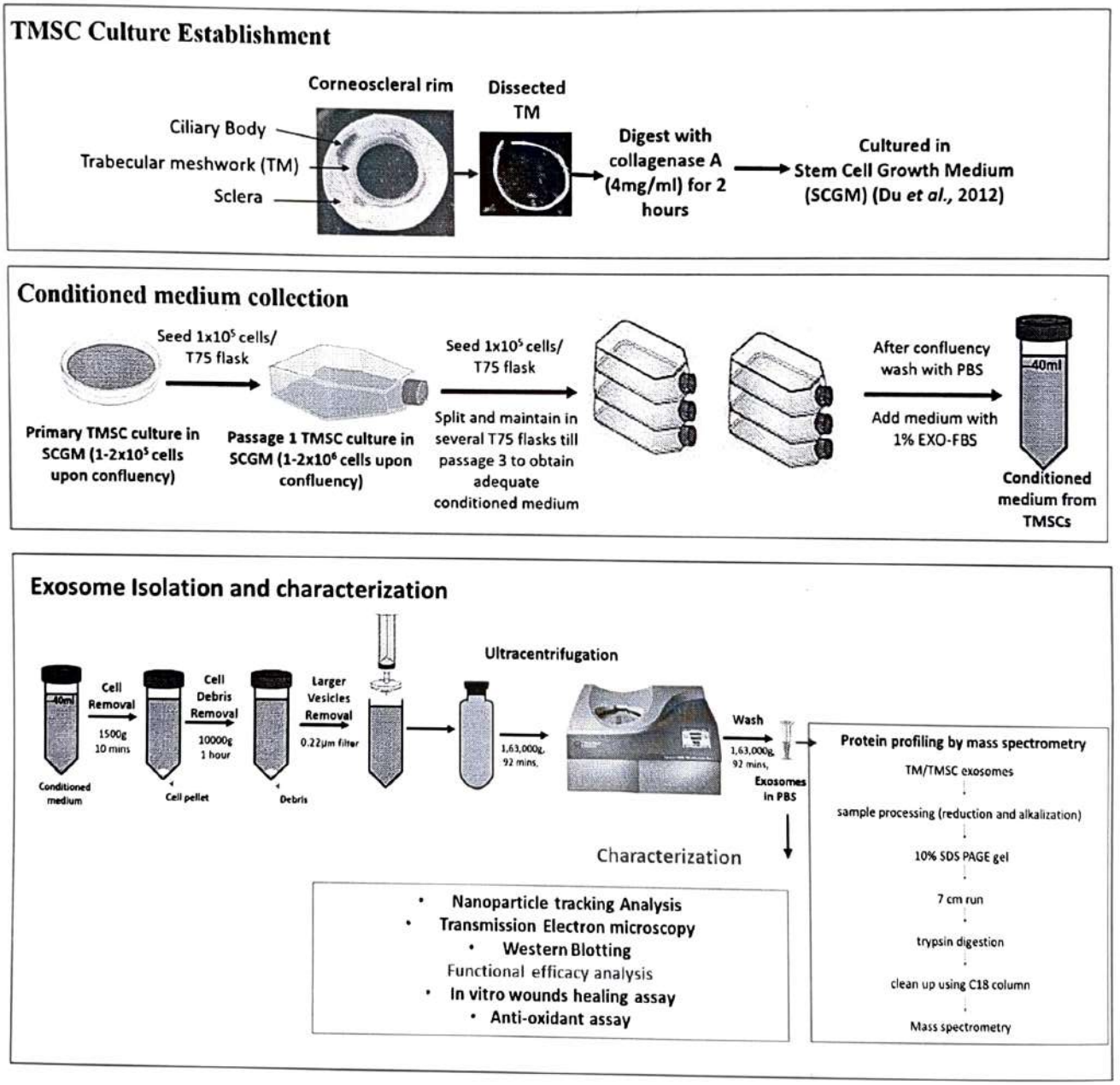
1. To isolate and characterize the exosomes from TM and TMSC conditioned medium.
2. To elucidate the potential of the TM and TMSC exosomes in TM regeneration *in vitro*
3. To decipher the molecular cargo of TM and TMSC exosomes by mass spectrometry.

Materials and Methods:

Sample collection

Whole globes that are not suitable for transplantation or corneoscleral rims after transplantation were collected from Rotary Arvind International Eye Bank, Madurai. The human tissues were handled according to the tenets of the Declaration of Helsinki and were approved by the Institutional Ethics Committee as well as Institutional Committee for Stem Cell Research (RES2018157BAS).

Graphical representation of over all work flow



Functional efficacy evaluation of TM/TMSC exosomes in vitro:

To evaluate the efficacy of exosomes in wound healing *in vitro* wound healing assay was performed by creating scratch in a monolayer culture using pipette tips to assess effect in migration was performed. To evaluate the anti-oxidant potential chronic oxidative stress in

TM cells was created using hydrogen peroxide (H2O2) after incubation MTT assay was carried out.

Results

Characterization of cultured TM and TMSCs

TM tissue was digested with collagenase and cultured in TM medium (Stamer *et al.*, 1995) and TMSC medium (Stem Cell Growth Medium-SCGM, Du *et al.*, 2012). Immunostaining for Mesenchymal stem cell (MSC) markers CD 90 and 105 revealed the presence of double positive cell in both TM medium and TMSC medium. However the stem cell content reduced upon passaging in TM medium and it was maintained in TMSC medium. The double positive expression of universal stem cell marker ABCG2 and neural crest stem cell marker p75 was absent when cells were cultured in TM medium while such cells were observed in TMSC medium (Table1).

Further to characterize the cells functionally, the TM and TMSCs were subjected to phagocytosis and sphere-forming assay. Both TM and TMSCs had the phagocytic activity which is essential to maintain outflow homeostasis in the anterior chamber. Sphere formation assay revealed that the TMSCs had 10-fold more functional efficacy compared to TM cells even though they express MSC markers. Together these results indicate the TM medium facilitates differentiation, while SCGM maintains TMSCs with functional efficacy.

Property	TM media	SCGM
Stem cell content	**Cells with high ABCG2 and p75** P0 = 0% **Cells with CD90 and CD105** P0=68±11.5, P1=47±4.58, P2=28.8±5.7, P3=22.2±5.9	**Cells with high ABCG2 and p75** P0 = 65.6±6.68% P1 = 12.76±1.26% P2 = 4.43±1.3% P3=2.7±0.5% **Cells with CD90 and CD105** P0=80.9±4.3, P1=79.8±7.75, P2= 74.9±3, P3= 58.6±2.4
Sphere forming ability	0.02±0.01%	0.24±0.1%
Phagocytic ability	Positive	Positive

Table1: Characteristic features of TM cells cultured in TM medium and SCGM

Characterization of exosomes

Exosomes from TM and TMSC conditioned medium was isolated by ultracentrifugation and characterized. The NTA analysis of isolated exosomes revealed the particle concentration from TM cultures to be $1.13 \times 10^{11} \pm 4.87 \times 10^{10}$ with a diameter of 164.5 +/- 31.5nm and TMSC cultures to be $1.6 \times 10^{11} \pm 1.35 \times 10^{10}$ with the diameter value of 162.0 +/- 8.5nm from approximately 42 million cells. This indicates 2696± 1160 particles per cell in TM and 3797±322 particles per cell in TMSC. The TM and TMSC exosomes were Poly dispersive with spherical shapes in TEM images (Figure 3). The common exosomal marker Syntenin

and TM exosomal marker neuropilin expression was observed in both TM and TMSC exosomes while emilin expression was only observed in TM exosomes by western blotting.

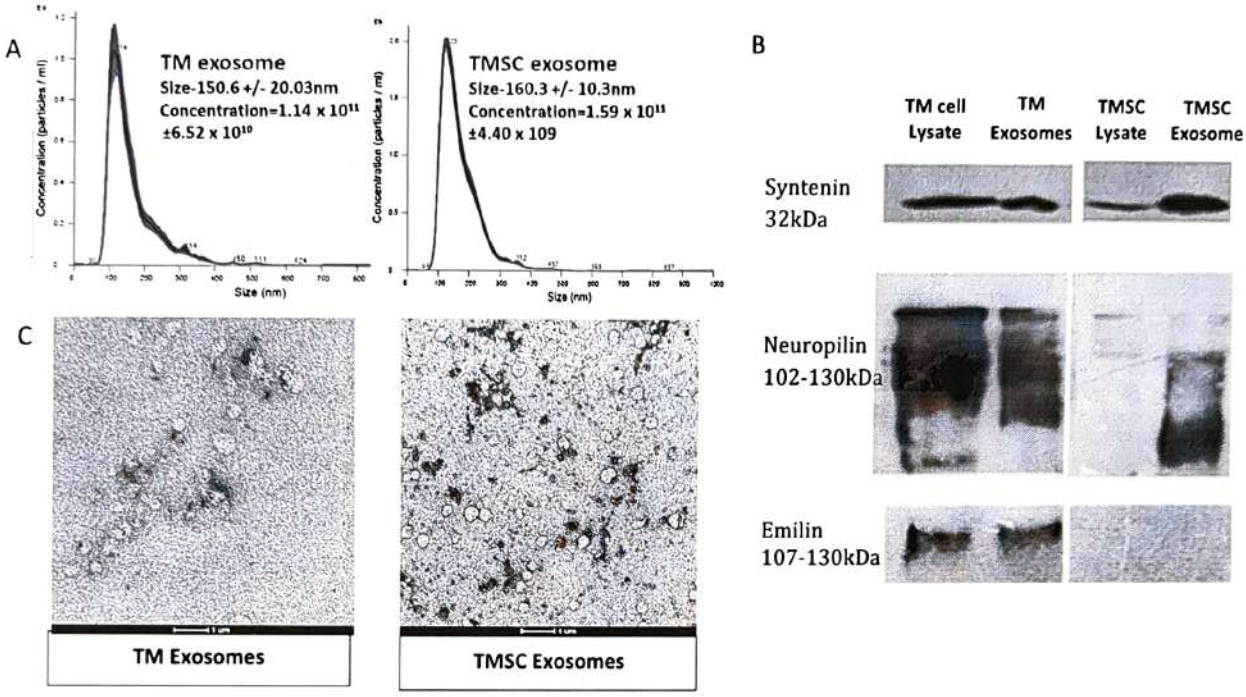


Figure 1: Characterization of isolated TM and TMSC exosomes- A- NTA, B- western blotting, and C-TEM analysis

In vitro wound healing assay

In the wound healing assay, at 48 hours the percentage of wound closure was 75.80±4.62% in control while with TM exosome it was 79.34±9.26%. In case of TMSC exosome-treated groups, the wound closure was 96.47±2.95% and 94.95±3.50% closure was observed with the positive control (EGF treated) (Figure 2).

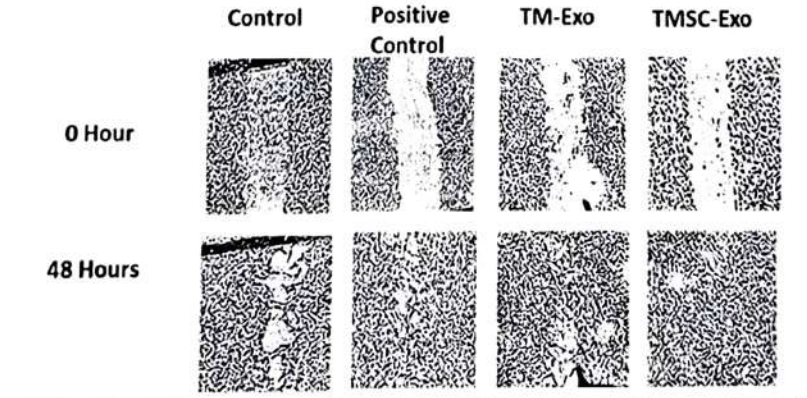


Figure 2: In vitro wound healing assay- representative inverted phase contrast microscopic images of TM cells at different time points after wound creation

The anti-oxidant potential of exosomes

A chronic oxidative stress in TM cells was established with H_2O_2 treatment. Upon treatment with 25µM and 50µM H_2O_2 the viability was 66.9± 1.33% and 63.23±3.82% respectively (Figure 3 A). 100µM H_2O_2 concentration resulted in 28.02±3.72% viability while with 200µM H_2O_2 viability was 4.2±3.42% (Figure 7A). With 25 and 50µM H_2O_2 there was higher viability, while with 200 µM H_2O_2 viability was drastically reduced which was not suitable to evaluate the efficacy of exosomes. Hence 100µM H_2O_2 concentration was used for further studies.

Upon TMSC exosome treatment the viability was 52.4 ±4.81% while with TM exosomes it was 30.6± 1.9% and 70.8± 8.3% when treated with positive control (ascorbic acid) in sham the viability was only 24.7±1.4% (Figure 3 B).

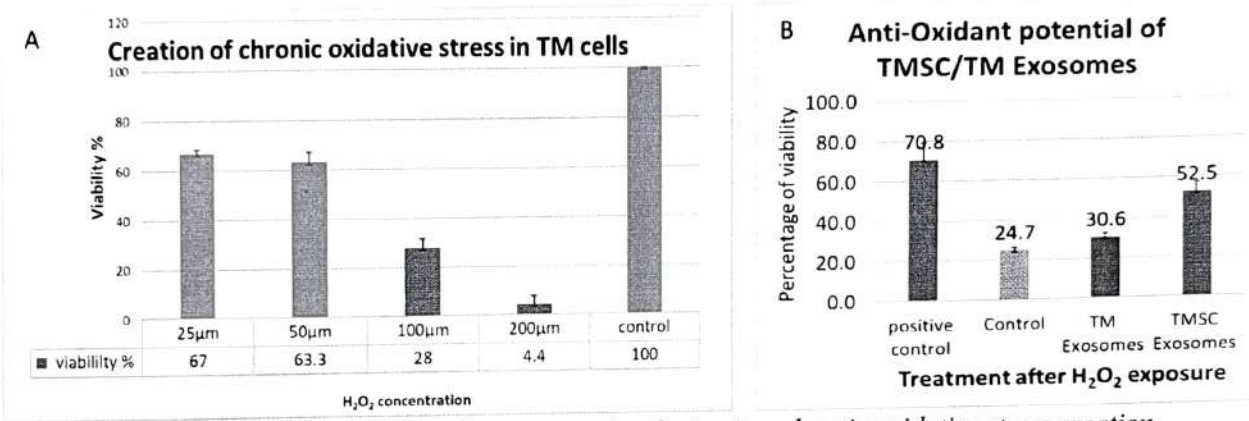


Figure 3 A- identification of H_2O_2 concentration for in vitro chronic oxidative stress creation, B- antioxidant efficacy evaluation of TM and TMSC exosomes

Protein profiling by Mass spectrometry

Proteomic analysis of exosomes identified with high confidence 1248 proteins in TMSC exosomes and 1328 in TM exosomes. 43.3% of the proteins were common between TM and TMSC exosomes while 30.9% proteins were only found in TMSC exosomes and 25.8% proteins only in TM exosomes. Bioinformatic analysis of the TM and TMSC exosomal protein revealed distinct profile of TM and TMSC exosomes (Figure 4).

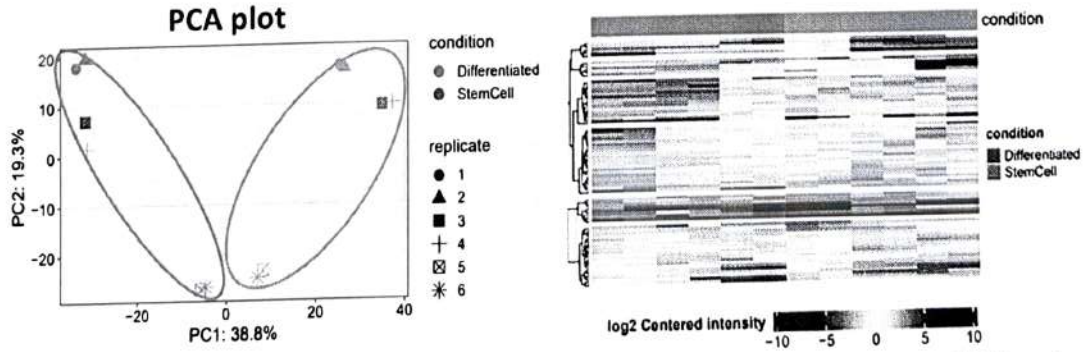


Figure 4: Representative PCA plot and heat map showing distinct protein profile of TM and TMSC exosomes

Functional prediction:

The identified TM and TMSC exosomal proteins were subjected to STRING analysis and the resulted proteins that are associated with wound healing and anti-oxidant function was compared with differential expression analysis data. Differential expression analysis revealed the up regulation of wound healing and anti-oxidant associated proteins in TMSC exosomes compared to TM exosomes (Figure 5).

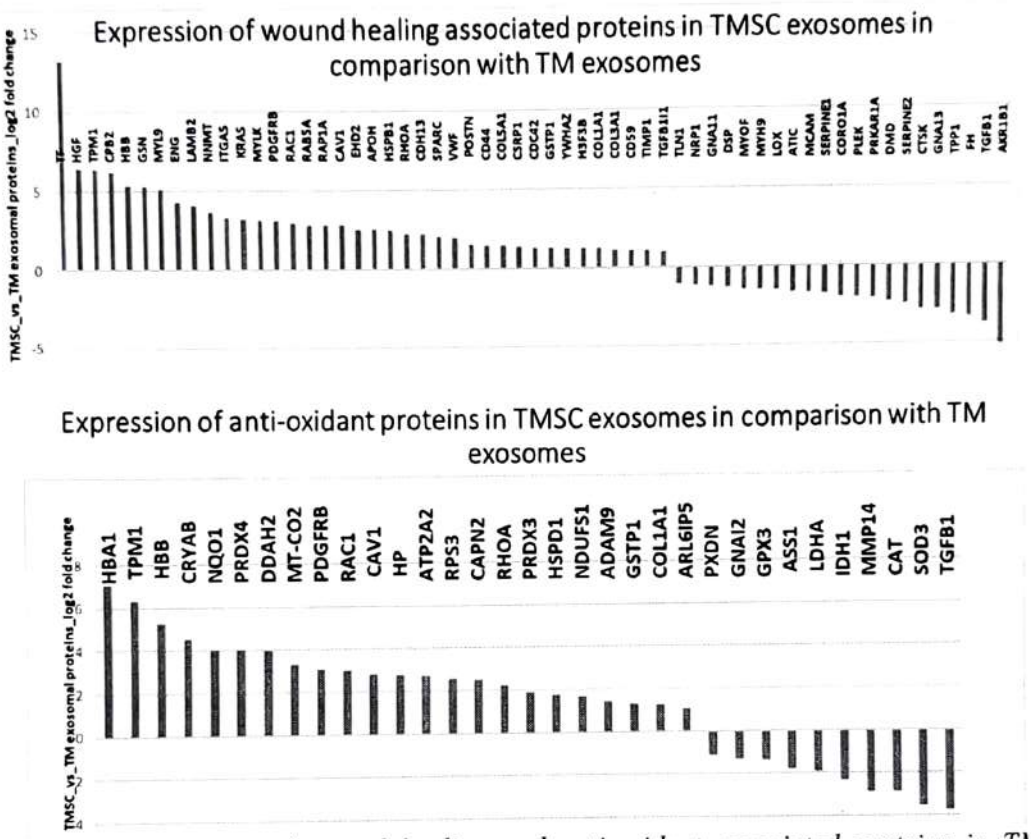


Figure 5: Expression of wound healing and anti-oxidant associated proteins in TMSC exosomes in comparison with TM exosomes

Discussion:

Previously, Du *et al.,* (2012) characterized the cells cultured in SCGM (TMSC medium) by flowcytometry, RT-PCR and immunocytochemistry and reported the abundance of CD90, CD73 and CD105 positive cells. Sundaresan et al., (2019) reported the absence of cells with high ABCG2 and p75 positivity in confluent TM cells cultured in TM medium. In this study, we have compared the phenotypic and functional properties of TM and TMSCs, which revealed in concordance with previous reports TM medium facilitates differentiation of TM cells while TMSC medium maintains stem cell with functional efficacy. Further exosomes from TM and TMSC conditioned medium was isolated and characterized. Characterization revealed the isolated exosomes were within the size range (30 -200nm) and spherical in nature. Stamer *et al.,* (2011), reported emilin and neuropilin to be TM exosome specific markers based on mass spectrometry data. Our western blotting data demonstrated the

presence of neuropilin in both TM and TMSC exosomes while emilin expression was restricted to TM exosomes.

Stem cell-derived exosomes have been reported to either induce or suppress cell proliferation based on the source cell and environment. For example, MSC exosomes are well-known to repair damaged tissues like cartilage, cutaneous wounds, ischemic myocardium, osteoporosis and corneal epithelial wounds by inducing cell proliferation (Zhang *et al.*, 2017, Shabbir *et al.*, 2015, Ju *et al.*, 2018, Zhao *et al.*, 2018, Han *et al.*, 2017, Zhou *et al.*, 2023) whereas in certain environment like cancer and proliferative diabetic retinopathy (Karaoz *et al.*, 2019, Xu *et al.*, 2020, Liang *et al.*, 2022) the exosome tend to inhibit cell proliferation. To elucidate efficacy of TMSC exosomes in regeneration, *in vitro* wound healing assay was carried out. The results indicated that The TMSC exosome treatment accelerates TM cell proliferation and wound closure compared to TM exosomes.

To address the oxidative stress in glaucomatous condition, chronic oxidative stress was created in TM cells *in vitro* based on previous reports (Li *et al.*, 2007, Luna *et al.*, 2009, Lin *et al.*, 2016). Different concentrations (25-200μM) of H_2O_2 were tested on TM cells hydrogen peroxide was used to identify the optimal concentration to create chronic oxidative stress. The lower concentrations (25 and 50μM) resulted in low oxidative damage hence the cell survival rate was higher (even in sham-data not shown). Chen *et al.*, reported the same phenomenon as the lower oxidative damage induces cellular endogenous antioxidant defence system the rate of survival/ recovery is increased (Chen *et al.*, 2005). With the higher concentration 200 μM, the cell viability was reduced by 96%. 100 μM H_2O_2 concentrations resulted in 28.02±3.72% optimal as previously reported to study the effect of exosomes (Li *et al.*, 2021). Li *et al.*, have demonstrated that pre-treatment with BMSC exosomes also enhances the TM cell survival under H_2O_2 (100 μM) induced oxidative stress. The BMSC exosome treatment reduced iROS production and inhibited the inflammatory cytokine in TM cells while MMPs were up regulated. We have demonstrated that, the TMSC exosomes had better anti-oxidant potential and promote TM cell survival under chronic oxidative stress compared to TM exosomes.

Huang *et al.*, (2017) compared bone marrow-derived MSC (BMSC) exosomes and umbilical cord-derived MSCs exosomes (UMSC) and demonstrated higher wound healing efficacy of BMSC is associated with its protein profile compared to UMSC. To identify the cargo responsible for TMSC exosomes functional efficacy mass spectrometry analysis was carried out. Bioinformatics analysis indicated the distinct protein profile between TM and TMSC exosomes with the up regulation of proteins associated with wound healing and anti-oxidant potential in TMSC exosomes.

Impact of the research in advancement of knowledge or benefit to mankind:

We have demonstrated that TMSC exosomes to have enhanced wound healing and anti-oxidant potential *in vitro*. Proteomic analysis also confirmed the up regulation of corresponding proteins in TMSC exosomes compared to TM exosomes. This established "a

proof of concept" for developing a TMSC exosome based therapy for patients with primary open angle glaucoma.

Primary open angle glaucoma is a leading cause of irreversible blindness worldwide and there are about 12 million people in India affected with glaucoma, accounting for 12.8% of blindness in our country. Current therapy for glaucoma is focused on reducing intraocular pressure by life-long use of drugs to reduce aqueous humour production or by increasing the outflow by surgical intervention. As an alternative to the above, our findings will enable the establishment of a nanovesicles based therapy for treating patients with glaucoma.

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