Effect of α-pinene and thymoquinone on the differentiation of bone marrow mesenchymal stem cells into neuroprogenitor cells

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Abstract
Introduction: Neurodegenerative diseases are accompanied by loss of neuronal function and integrity. Stem cell therapy is utilized to regenerate neurons to repair the damaged area. Regeneration potential of stem cells can be enhanced by using chemicals with known bioactive properties. In the current study, two bioactive compounds, α-pinene (AP) and thymoquinone (TQ) were explored for their neuronal differentiation potential of rat bone marrow mesenchymal stem cells (MSCs).

Methods: MSCs were isolated, cultured and characterized immunocytochemically for the presence of specific surface markers. Optimized concentrations of both compounds (20 µM AP and 12 µM TQ) as determined by MTT assay, were used to treat MSCs in separate and combined groups. All groups were assessed for the presence of neuronal, astroglial, and germ layer markers through qPCR. Neuronal and glial protein expression were analyzed by immunocytochemistry.

Results: Both compounds alone and in combination induced differentiation in MSCs with significant gene expression of neuronal markers i.e. neuron specific enolase (NSE), nestin, microtubule-associated protein 2 (MAP2), neurofilament light chain (Nefl) and Tau, and astroglial marker i.e. glial fibrillary acidic protein (GFAP). AP treated group also showed significant upregulation of endodermal and mesodermal markers indicating transition of ectoderm towards the other two germ layers.

Conclusion: This study concludes that AP and TQ potentially differentiate MSCs into neuronal and astroglial lineages. However, AP treated group followed germ layer transition. Expression of neuronal as well as glial markers indicate that the differentiated neurons are at the neuroprogenitor stage and can be potential candidates for cellular therapeutics against neurodegenerative disorders.

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class of monoterpenes. It suppresses inflammation and catabolism by modulating inflammatory reactions and by reforming the extracellular matrix in human chondrocytes. It has been reported to considerably reduce heart rate and artery-generated blood pressure in rats and this effect is triggered in the vasomotor center of the medullary region of brain and nucleus tractus solitarius. It also inhibits cellular proliferation and blood vessel formation. In yeast cells and mitochondria, AP and β-pinene disrupt cell membrane integrity, suppress respiration and ion transportation, alleviate membrane permeability and regulate various cellular functions, i.e. energy maintenance and other membrane-coupled energy transduction processes, solute transportation, and regulation of metabolic processes. AP significantly inhibits acetylcholine esterase, thus useful in treating cognition disorders. TQ is the major active component of Nigella sativa, present in its volatile and fixed oil. It possesses anti-oxidant, anti-inflammatory and other bioactive properties. It scavenges reactive oxygen species and neutralizes them, thus reducing their detrimental effects. TQ increases the expression level and action potential of glutathione S-transferase, glutathione peroxidase, superoxide dismutase and glutathione reductase. TQ has also been shown to reduce neuronal inflammation induced in response to NF-kB in BV-2 microglial cells. Given the remarkable biological properties of these two compounds, this study was designed to investigate the potential effect of AP and TQ and their combination on the differentiation of rat bone marrow derived mesenchymal stem cells (MSCs) into neuronal lineage at both gene and protein levels.

Material and Methods

Materials

The reagents/materials used in the study are as follows: Bright green 2x qPCR Master Mix (Applied Biological Materials Inc., Canada), CD44 and CD45 antibodies (BD Biosciences, USA), NeuN antibody (Biolegend, USA), CD90 antibody (Cedarlane Cellutions Biosystems, Canada), glial fibrillary acidic protein (GFAP) antibody (Cloud Clone, USA), bovine serum albumin, DAPI, Tween-20 (MP Biomedicals, USA), β-III tubulin antibody (R&D Systems, USA), Alexa fluor 546 rat anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., USA), AP, MTT, TQ, Triton X-100, Vimentin antibody (Sigma Aldrich, USA), c-kit (CD117) antibody, DMEM, L-glutamine, penicillin-streptomycin, RevertAid First Strand cDNA synthesis kit, Sodium pyruvate, Trypsin EDTA (Thermo Fisher Scientific, USA).

Experimental animals

Male adult Wistar rats, weighing 170-200 g, were used to isolate bone marrow MSCs. These animals were provided by the in-house facility of the institute. They were kept at a temperature of 24 ± 1°C at relative humidity of 55 ± 5%, with 12/12 hours light /dark cycle. Sawdust was used as bedding and animals were given access to water and food during the entire study period.

Culture of bone marrow MSCs

Animals were sacrificed. Tibia and femur were dissected and muscles were removed. All the cell culture work was performed in a sterile biosafety cabinet (ESCO, USA). Bone ends were removed and marrow was flushed through bone cavities by means of a 26-gauge needle filled with complete medium (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin and 1 mM sodium pyruvate). After thorough and gentle mixing, marrow cells were transferred to T-75 cm² flasks and incubated at 37°C in humidified CO₂ incubator. Medium was changed after every 3 to 4 days. Once cells attained about 80% confluence, they were sub-cultured using 1X trypsin. Passage 1 and 2 (P1 and P2) cells were used for all experiments.

Characterization of MSCs by immunocytochemistry

MSCs were cultured in 24-well plate and incubated at 37°C for 24 hours. The next day, medium was removed from wells and cells were washed with PBS twice. Paraformaldehyde (4%) with a volume of 200 µL was added to each well for 10 minutes followed by washing with PBS. Triton X-100 (0.1%) was added and plates were incubated at room temperature for 10 minutes followed by washing with PBS. Blocking solution (2% BSA, 0.1% tween 20 in PBS) was then added and the plate was proceeded for incubation at room temperature for 30 minutes. Primary antibodies i.e. CD44, CD90, CD117, CD45 and vimentin were added to their respective wells, followed by overnight incubation at 4°C. Primary antibody solutions were then removed and wells were washed with PBS. Alexa flour 546 goat anti-rat secondary antibody was added to each well and incubated for 2 hours at 37°C. Following washing with PBS, DAPI (0.5 µg/mL) was added for 15 minutes at room temperature. Wells were washed again and cells were analyzed under fluorescence microscope (Nikon, Japan).

Cytotoxicity analysis

Cytotoxicity analysis of AP and TQ was performed by MTT assay to find out the optimum and safe concentration for further experiments. MSCs were seeded in 96-well plate and incubated at 37°C in humidified 5% CO₂ incubator for 24 hours. Confluent MSCs in each well were treated with different concentrations of both compounds separately (AP 5-60 µM; TQ 3-18 µM) and incubated further for 24 hours. The medium was removed from the wells and MTT dye was added followed by incubation under standard culture conditions for 4 hours. Dye was removed,
DMSO was added and the plate was read at 570 nm using spectrophotometer (Multiskan Go, Thermo Scientific, USA). Optimized concentrations of each compound were further analyzed in combination for cytotoxicity analysis using similar experimental setup.

**Experimental groups**

MSCs were categorized into four groups; control (MSCs without treatment), MSCs treated with 20 µM AP and 12 µM TQ separately, and in combination for the analysis of the synergistic effect.

**Treatment of MSCs**

Working solutions of 20 µM AP and 12 µM TQ were prepared using their corresponding stocks in serum-free DMEM. MSCs were treated with the compounds separately and in combination. The control group was not given any treatment. After treatment, MSCs corresponding to all groups were incubated at 37°C in the humidified 5% CO₂ incubator for 3 hours.

**Analysis of gene expression by qPCR**

RNA was isolated from each group by TRIzol method according to the manufacturer’s instructions. The yield was measured using NanoDrop UV-Vis spectrophotometer (Thermo Scientific, USA). cDNA synthesis was performed using RevertAid kit according to manufacturer’s protocol. Gene expression levels of neuronal, glial, and germ layer markers were analyzed in each group by qPCR (Mastercycler, ep realplex, Eppendorf, Germany) using qPCR Master Mix. Primer sequences corresponding to these genes are presented in Table 1. Reaction was performed with initial denaturation at 95°C for 10 minutes followed by 40 cycles (denaturation at 95°C for 15 seconds and annealing at 58°C for 1 minute). The Ct values obtained after completing the whole reaction were used to calculate the fold change.

**Analysis of neuronal and glial protein expression**

Immunocytochemical analysis of treated cells was performed to determine the expression level of GFAP, NeuN, and β III tubulin proteins using fluorescence microscope. The intensity of immunocytochemically analyzed images was calculated via Imagej software.

**Statistical analysis**

Data of each experiment was analyzed statistically by IBM-SPSS Statistics software (v22.x86-EQUINOX). Independent samples t test or one-way ANOVA was used for analysis. All values were represented as mean ± SEM with number of observations (n) = 3 and level of significance = P < 0.05.

**Results**

**MSCs culture and characterization**

Bone marrow culture comprised of heterogeneous population of cells. MSCs showed adherence to the flask surface unlike the floating hematopoietic stem cells which were removed. After about 2 weeks, MSCs proliferated and formed a monolayer, presenting fibroblast-like morphology. Confluent MSCs were sub-cultured to passage 1 and 2 (Fig. 1A).

MSCs were characterized by immunocytochemistry based on the presence of cell surface markers. Cells showed positive expression of CD44, CD90, CD117, and vimentin proteins (Fig. 1E-H) comparing to the control (Fig. 1D), while CD45, a hematopoietic marker, was not expressed in these cells (Fig. 1I).

**Cytotoxicity analysis**

Cytotoxicity of AP and TQ was evaluated by MTT assay. There was a concentration dependent increase in the number of metabolically inactive cells. AP and TQ showed statistically significant toxic effect on concentrations above 40 µM and 12 µM, respectively (Fig. 2A-B). Based on these results, AP and TQ at

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**Table 1. Neuronal, glial and germ layer transition genes**

| Neuron Specific Genes | Sequence |
|-----------------------|----------|
| Nestin                | Forward: AACCACAGGAGTGGGAACTG  
Reverse: TCTGGCATTGACTGAGCAAC |
| Nefl                  | Forward: GCCGAAGGAGTGGTTCAAGAG  
Reverse: TGTCTGCATTGCTGTGACCC |
| NSE                   | Forward: GTGGACACACATCAACAGCAC  
Reverse: TGAGCAATGTGGCGATAGAG |
| MAP2                  | Forward: ACCTGGCTGGACTCAATACC  
Reverse: ATGGAGCAAAACCAAAGG |
| Tau protein           | Forward: CACTGCCGTCCATCAACAGGA  
Reverse: ATGGAGCAAAACCAAAGG |

| Glial Specific Gene  | Forward: AGAAACCGCCTACACCAGTC  
Reverse: GCACACCTACATCACATCC |

| Germ Layer Transition Genes |
|----------------------------|
| Ectodermal Markers |
| NCAM | Forward: CACCGACAGGAGGACGGA  
Reverse: CCACATGGAAGTCTCCACCAG |
| Nestin | Forward: AACCACAGGAGTGGGAACTG  
Reverse: TCTGGCATTGACTGAGCAAC |
| Pax6 | Forward: CGGGAACACTCTCAACAGGA  
Reverse: TGGCTTCTGTAGCAAAAGGT |

| Endodermal Markers |
|---------------------|
| AFP | Forward: GCCCTGAATGACAGAGGAGGCA  
Reverse: CCACATGGAAGTCTCCACCAG |
| SOX17 | Forward: CACCGCAGTAGGCGATCTC  
Reverse: CAGCATGGAAGTCTCCACCAG |
| MixL1 | Forward: GGGCTGTGACATTTGAACTCATC  
Reverse: GTGCTTCTGCAGAGCTTTAGGT |

| Mesodermal Markers |
|-------------------|
| Mesp1 | Forward: GGACCCATCATCTCTGTTGACCC  
Reverse: TCCACAGGAAATCCCCCTCTC |
| T Brachyury | Forward: GCTGGTGAGGAGGAGGCTCA  
Reverse: GAAACATCCTCCTGGCTCCTT |
| Tbx20 | Forward: ACTGACATCGAGGAGGAGTAA  
Reverse: CTGTGAGGAGGAGCTTCATAGC |

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concentrations of 20 µM and 12 µM, respectively were used for further experiments. When both compounds were used in combination and their combined effect was analyzed, it also appeared to be non-toxic to the MSCs (Fig. 2C).

Morphological changes in treated MSCs
Treated MSCs were monitored periodically to observe changes in their morphology. Distinguished features of neuronal cells started to appear within 1 hour after the treatment in each group. Prominent neuronal morphology was observed with formation of distinctive cell body and dendrites (Fig. 3A).

Neuronal and glial gene expression analysis in treated MSCs
MSCs after 3 hours of treatment were evaluated for the expression of neuronal genes by qPCR. Each group showed significant upregulation in the expression of neuron specific genes, neuron specific enolase (NSE), MAP2, Tau, Nestin and neurofilament light chain (Nefl) as compared to the control (Fig. 3B). Treated MSCs were further analyzed for the expression of the astroglial specific marker, GFAP which also showed significant over expression in all the treatment groups (Fig. 3C).

Neuronal and glial protein expression analysis in treated MSCs
Treated MSCs were analyzed by immunocytochemistry for the expression of neuronal and glial proteins. Expression levels of GFAP, NeuN and β-III tubulin were found to be significantly increased in all treatment groups as compared to the control (Fig. 4).

Analysis of germ layer markers
To check whether AP and TQ treatment also affect meso-

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Fig. 1. Morphology and characterization of rat bone marrow derived MSCs: Passage 1 MSCs showing (A) fibroblast-like morphology and (B) DAPI stained nuclei. (C) Also shown is the superimposed image of A and B. Characterization of MSCs by immunocytochemistry showing (D) unlabeled control, (E-H) positive expression of CD44, CD90, CD117 and vimentin, respectively, and (I) negative expression of CD45, a hematopoietic marker.

Fig. 2. Cytotoxicity analysis of α-pinene (AP), thymoquinone (TQ), and the combination (AP + TQ) group by MTT assay: (A) AP above 40 µM and (B) TQ above 12 µM concentrations showed statistically significant reduction in cell survival. (C) Combined treatment of AP and TQ (20 and 12 µM, respectively) did not show significant cytotoxic effect on MSCs. Data is presented as mean ± standard error with n = 3 and significance level *P < 0.05, **P < 0.01 and ***P < 0.001.)
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and endodermal lineage commitment, genes specific to endodermal (AFP, Sox17 and MixL1) and mesodermal markers (Mesp1, Tbx20 and T Brachyury) were analyzed along with the ectodermal markers (Nestin, Pax6 and NCAM). As expected, all treatment groups showed increase in the expression of ectodermal markers. For the other two germ layer markers, AP treatment showed significant increase in all endodermal and mesodermal markers, while TQ and the combined treatments showed significant upregulation of Tbx20 gene only as compared to the control (Fig. 5).

Discussion

Stem cell differentiation into various lineages can be triggered in response to various inducing agents i.e. growth factors, cytokines, peptides, small molecules etc. These inducers particularly target a pathway, directing stem cells towards a certain lineage or cell type. Current study is...
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performed to explore the potential role of small molecules i.e. AP and TQ in the differentiation of bone marrow derived MSCs towards neuronal lineage. These molecules possess a variety of pharmacological and biological properties and are known to have many beneficial effects. We used rat bone marrow MSCs and treated them with these compounds in order to differentiate these cells into neuronal lineage. Prior to the treatment, optimized concentration of these compounds was selected based on the cytotoxicity analysis. AP at 20 µM and TQ at 12 µM concentrations were found to be non-toxic to MSCs and therefore utilized for all subsequent experiments. MSCs were divided into four experimental groups i.e. untreated MSCs (control) (1), MSCs treated with 20 µM AP (2) and 12 µM TQ (3) separately, and in combination (4). All the groups showed differentiation of MSCs towards neuronal lineage within 1 hour of treatment showing apparent neuronal morphology with very prominent cell body and dendrite formation. After 3 hours of treatment, analysis of neuronal gene expression in differentiated MSCs showed that there was significant upregulation in the expression of neuron specific markers i.e. NSE, Tau, nestin, Nefl, and microtubule-associated protein 2 (MAP2) in all the treated groups compared to the untreated control, showing neuronal differentiation at the gene level.

We also analyzed astroglial specific gene i.e. GFAP which also revealed significant upregulation in all the treated groups. Co-expression of glial marker along with the neuronal markers have also been demonstrated in other studies. Differentiation was also verified at protein level using immunocytochemistry. All the treated groups showed intense expression of GFAP, β-III tubulin and NeuN proteins, confirming the neuronal and glial differentiation at the protein level as well.

The morphological features of neurons observed in the treated groups retained for about 4-5 hours, after which the differentiated MSCs reverted to their fibroblast like morphology especially in case of AP. This implies that neural differentiation is maintained for an initial time period, and later the cells either de-differentiated or underwent lineage transition from ectodermal to mesodermal or endodermal germ layer. To confirm this, we selected specific genes corresponding to each germ layer and analyzed their expression through qPCR. mRNA analysis revealed the upregulation of ectodermal markers (Nestin, Pax6 and NCAM), which is expected as the neuronal cells are derivatives of ectodermal germ layer. However, we also observed significant upregulation of endodermal (AFP, Sox17 and MixL1) and mesodermal (Mesp1, T Brachyury and Tbx20) markers in AP treated group. The upregulation of T Brachyury and MixL1 genes represent the reversal of differentiated neurons towards fibroblast-like cells as the overexpression of these genes have been reported in the development of endodermal and mesodermal layers. These outcomes are supported by other studies that validate the upregulation of MixL1 gene on primitive streak during embryonic development, which is responsible for the formation of mesodermal and endodermal germ layer. This gene governs the development of germ layers and behaves as a crucial regulatory molecule.

The process of development of all three germ layers (gastrulation) is considered as the most prominent gestational phase. Gastrulation is followed by the formation of primitive streak on the epiblast region. The cells of the epiblast migrate towards the primitive streak.
and adopt flask shaped appearance. The cells become detached from the epiblast and invaginate. Some of these cells replace the hypoblast and form the endoderm while others move between the newly formed endoderm and epiblast, resulting in the formation of mesoderm. During this phenomenon, T Brachury gene is expressed in the node, precursor cells of notochord that aids the cells to migrate through primitive streak and causes the formation of mesodermal layer.26

Presence of all the three germ layer markers have been documented in previous studies on MSCs in their uncommitted state,27 so it is possible that these MSCs differentiate into any lineage depending upon the characteristic of the inducer. It is also possible that the inducer is not specific towards a particular lineage and therefore MSCs follow the normal transition in the development of germ layers, finally resulting in the formation of cells of various lineages. With these findings, it is the most likely explanation in case of AP, while in case of TQ and the combination group, cells were likely to differentiate into both neuronal and glial lineages implying that the differentiation is towards the neuroprogenitor stage.26

Conclusion
The findings of the current study demonstrate that AP and TQ efficiently induced differentiation of rat bone marrow derived MSCs towards neuronal lineage. These neuronal cells expressed astroglial marker, which either indicates that both compounds possess differentiation potential towards both lineages or implies that the differentiation is at the neuroprogenitor stage. Furthermore, AP treatment results in the transition of germ layer from ectoderm to the endoderm and mesoderm, as specific markers were found to be overexpressed. Further studies are needed to explore the potential of these compounds so that the differentiated neuroprogenitors can be used for the management of neurodegenerative ailments.

Research Highlights

What is the current knowledge?

✓ AP and TQ possess remarkable biological properties of medicinal importance.
✓ AP and TQ also serve as neuroprotective agents

What is new here?

✓ AP and TQ induced neuronal differentiation with marked expression of astroglial specific gene, GFAP in rat bone marrow MSCs.
✓ Induction by these compounds can be considered at the neuroprogenitor level.
✓ AP induced MSCs followed germ layer transition (from ectoderm to endoderm and mesoderm) as the Mix1I and T Brachury genes are markedly overexpressed.

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Ethical statement
All experiments were performed as per the institutional policy in accordance with the NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC). Protocol no. 2018-0014 was assigned to the study.

Competing interests
The authors declare no conflict of interest.

Authors’ contribution
AS and DSHA conceived the idea and reviewed the manuscript; IK and AS designed the experiments; AI, RQ and TSM performed experiments. AI wrote the first draft of the manuscript and analyzed the data. IK and AS analyzed and interpreted the data; AS edited and finalized the manuscript.

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AS and DSHA conceived the idea and reviewed the manuscript; IK and AS designed the experiments; AI, RQ and TSM performed experiments. AI wrote the first draft of the manuscript and analyzed the data. IK and AS analyzed and interpreted the data; AS edited and finalized the manuscript.

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