褪黑激素体外对脂肪间充质干细胞增殖及向施万样细胞分化的影响

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摘要 该文探究了褪黑激素(melatonin, MT)对脂肪间充质干细胞(adipose mesenchymal stem cells, ADSCs)向施万细胞(Schwann cells, SCs)分化的影响。从小鼠腹股沟脂肪垫分离ADSCs, 培养至第3代, 进行成脂和成骨分化, 流式细胞术鉴定细胞表面抗原; 第3代ADSCs分别与不同浓度的MT孵育24 h, 采用CCK-8法检测细胞增殖活性, 确定MT合适浓度。实验分为对照组、50 nmol/L MT组、神经诱导液组和50 nmol/L MT+神经诱导液组, 孵育11天后, 采用qPCR和Western blot检测SCs表面标志GFAP和S-100的表达情况。结果显示, 50和100 nmol/L MT对ADSCs的增殖活性显著高于其他组, 且50 nmol/L MT促增殖效果更好。神经诱导液组和50 nmol/L MT+神经诱导液组GFAP、S-100的mRNA和蛋白表达水平均显著高于对照组, 而与神经诱导液组相比, 50 nmol/L MT+神经诱导液组GFAP、S-100的mRNA和蛋白表达水平显著升高。综上所述, 50 nmol/L MT可以在体外显著促进ADSCs增殖, 同时MT和神经诱导液协同作用后, 可显著促进ADSCs向SCs分化。

关键词 褪黑激素; 脂肪间充质干细胞; 施万细胞; 增殖; 分化

Melatonin Regulates Proliferation of ADSCs and Differentiation of ADSCs to SCs *in vitro*

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Abstract The purpose of this study was to investigate the effect of MT (melatonin) on the differentiation of ADSCs (adipose mesenchymal stem cells) into SCs (Schwann cells). ADSCs were isolated from mouse inguinal fat pad and cultured to the third generation for adipogenic and osteogenic differentiation. Cell surface antigens were identified by flow cytometry. The third-generation ADSCs were incubated with MT at different concentrations for 24 h, and the proliferation activity of cells was detected by the CCK-8 method, and then the appropriate concentration of MT was determined. The experiment was divided into control group, 50 nmol/L MT group, neural induction fluid group, and 50 nmol/L MT+neural induction fluid group. After 11 days of culture, the expression of GFAP and S-100 on the surface of SCs was detected by qPCR and Western blot. The results showed that the proliferation activity of 50 and 100 nmol/L MT on ADSCs was significantly higher than that of other groups, and the proliferation effect of 50 nmol/L MT was better. The mRNA and protein expressions of GFAP and S-100 in the nerve induc-

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tion fluid group and 50 nmol/L MT+nerve induction fluid group were significantly higher than those in the control group, while the mRNA and protein expressions of GFAP and S-100 in the 50 nmol/L MT nerve induction fluid group were significantly higher than those in nerve induction fluid group. In conclusion, 50 nmol/L MT can significantly promote the proliferation and differentiation of ADSCs *in vitro*.

Keywords melatonin; adipose mesenchymal stem cells; Schwann cells; proliferation; differentiation

周围神经损伤(peripheral nerve injury, PNI)是周围神经干及分支受到外界直接或间接作用而引起的损伤, 在临床上较为常见。施万细胞(Schwann cell, SCs)是构成周围神经轴突髓鞘的重要细胞, 可分泌神经营养因子促进轴突再生, 在PNI再生中至关重要[1-3]。然而, SCs来源有限, 自我增殖能力弱, 自体取材还会导致供区相应功能损伤, 临床应用存在局限性。

脂肪间充质干细胞(adipose mesenchymal stem cells, ADSCs)是一种多能干细胞, 不仅可以分化为脂肪细胞、成骨和软骨细胞、神经表型及SCs[4-5], 还具有来源丰富、增殖率高、免疫耐受等临床优势, 因此, ADSCs是一种替代SCs治疗PNI的方法。然而, 研究表明, 由于微环境的影响, ADSCs在向SCs分化时, 效率较低, 仅能达到ADSCs的30%~70%, 难以达到预期效果[3,6]。因此, 提高ADSCs向SCs的分化效率是PNI修复的关键。

褪黑激素(melotonin, MT)主要由松果体分泌, 具有抗氧化、抗炎和抗凋亡等生物学功能, 可保护神经细胞免受损伤或死亡[7-8], 且在细胞增殖和分化中也发挥着重要作用。以往的研究表明, MT具有促进胚胎干细胞增殖[9]、诱导多能干细胞分化为神经细胞[10]、促进人牙髓细胞分化为肝细胞等功能[11]。我们之前的研究证明, MT可以通过MT受体和下游MEK/ERK1/2通路增加PC12细胞分化的神经元数量[12]。

虽然已被证实MT在各种细胞的增殖和分化中发挥积极作用, 但MT调控ADSCs向SCs分化的报道鲜少。因此, 本研究旨在探讨MT在体外对ADSCs增殖和向SCs分化的影响。

1 材料与方法

1.1 实验动物和主要试剂

3周龄昆明小鼠由河南科技大学实验动物中心提供(该实验伦理批准号为SDXN2019010)。I型胶原酶、MT、油红O、茜素红、抗小鼠FITC标记的单克隆抗体、Trizol试剂购自美国Sigma公司; FBS购自浙江天航生物科技有限公司; DMEM/F12购自美国HyClone公司; CCK-8试剂盒、RIPA、BCA试剂盒购自上海碧云天生物技术有限公司; Cocktail购自北京鼎国长生生物技术有限公司; GFAP兔多克隆抗体、S-100兔多克隆抗体、二抗购自Proteintech公司。

1.2 ADSCs的分离和培养

无菌条件下取小鼠腹股沟脂肪组织, 充分用眼科剪刀机械剪碎后, 于0.1% I型胶原酶消化60~80 min (37 ℃)。分离的细胞于添加10%胎牛血清、100 U/mL青霉素、100 mg/mL链霉素的DMEM/F12培养基中, 在37 ℃、5% CO_2条件下培养。待细胞达到80%~90%融合时, 进行消化传代。

1.3 ADSCs的成脂诱导

将第2代的ADSCs消化后, 按5×10^{5}个/mL密度置于6孔板中。ADSCs培养至80%融合时, 在含10%胎牛血清、异丁基甲基黄嘌呤(0.5 mmol/L)、地塞米松(10^{-6} moL/L)、胰岛素(10 mg/L)、吲哚美辛(0.2 mmol/L)的DMEM/F12成脂诱导培养液中培养14天, 然后, 用油红O染色液对固定的细胞进行染色, 并在倒置显微镜下观察结果。

1.4 ADSCs的成骨诱导

将第2代的ADSCs消化后, 按5×10^{5}个/mL密度置于6孔板中。ADSCs培养至80%融合时, 在添加10%胎牛血清的DMEM、地塞米松(10 nmol/L)、抗坏血酸-2-磷酸(50 μg/mL)和β-甘油磷酸(10 mmol/L)的成骨诱导培养液中培养21天。然后, 用茜素红染色液对固定的细胞进行染色, 并在倒置显微镜下观察结果。

1.5 ADSCs表面标记物的检测

采用流式细胞术分析第3代ADSCs免疫表型CD34、CD44、CD45和CD90的表达。PBS(4 °C)洗涤2次后, 细胞与抗小鼠FITC标记的单克隆抗体在黑暗中室温孵育30 min。PBS冲洗后, 流式细胞仪分析。细胞染色时将不带FITC标记的IgG作为阴性对照。

1.6 CCK-8法检测ADSCs增殖情况

将第2代ADSCs(5×10^{5}个/孔)接种于96孔板中, 每

表1 引物序列

Table 1 Primer sequence

基因 Gene	上游引物(5′→3′) Forward primer (5′→3′)	下游引物(5′→3′) Reverse primer (5′→3′)
GFAP	5′-GTT ACC AGG CAC TTG CT-3′	5′-ACA GGA ATG GTG ATG CGG TT-3′
S-100	5′-AGG TCG TAG GGA AAG ACG A-3′	5′-ATT TCA GCA GCA CAC GGT TG-3′
β-actin	5′-CTA CCT CAT GAA GAT CCT GAC C-3′	5′-CAC AGC TTC TCT TTG ATG TCA C-3′

组设置5个重复, 待细胞长至80%时, 分别用含有不同浓度(0 nmol/L、10 nmol/L、50 nmol/L、100 nmol/L、200 nmol/L、300 nmol/L、500 nmol/L、1 000 nmol/L) MT的诱导液于37 °C孵育24 h。根据CCK-8试剂盒说明书, 每孔添加10 μL CCK-8溶液, 继续在37 °C孵育4 h。使用酶标仪于450 nm波长处测量各孔吸光度值。

1.7 ADSCs向SCs的诱导分化

第3代ADSCs分为4组: 对照组、50 nmol/L MT组、神经诱导液组和50 nmol/L MT+神经诱导液组。对照组的培养液为DMEM/F12培养液; 50 nmol/L MT组的培养液为含有50 nmol/L MT的DMEM/F12培养液; 神经诱导液组的培养液为神经诱导液; 50 nmol/L MT+神经诱导液组为含50 nmol/L MT的神经诱导液。诱导分化过程: 将第3代的ADSCs在补充β-巯基乙醇(1 mmol/L)的DMEM/F12中培养24 h, 然后于补充10% FBS和全反式维甲酸(35 ng/mL)的DMEM/F12中培养24 h。PBS洗涤后, 在37 °C的施万细胞诱导培养基中培养12天, 培养基为添加10% PBS、5 μmol/L佛司克林、5 ng/mL血小板源性生长因子-AA、10 ng/mL碱性成纤维细胞生长因子和200 ng/mL重组蛋白的DMEM/F12培养液[13-14]; 每隔2天换液1次, 然后在倒置显微镜下观察细胞形态变化。对照组和50 μmol/L MT组细胞的培养时间与其他两组细胞相同。

1.8 免疫荧光分析

ADSCs用神经诱导液诱导14天后, 用4%的多聚甲醛室温下固定20 min; PBS冲洗后, 用0.5% Triton X-100透化10 min; 使用5% BSA液室温封闭细胞30 min, 阻断非特异性结合位点。一抗S-100兔多克隆抗体(1:500), 于4 °C孵育细胞过夜; 二抗与FITC在37 °C下结合1 h; DAPI在黑暗中室温孵育5 min。抗荧光淬灭剂封片液封片后, 通过荧光显微镜观察细胞。

1.9 RNA提取和qRT-PCR

各组ADSCs总RNA的提取使用Trizol试剂。qPCR反应体系为: 5 μL SYB Green Mix、1 μL上游引物、1 μL下游引物、1 μL稀释cDNA、2 μL DEPC-H_2O组成。引物序列见表1。qPCR反应条件为: 95 °C预变性5 min; 95 °C变性15 s, 60 °C退火15 s, 72 °C延伸15 s, 循环45次。采用$2^{-\Delta\Delta Ct}$法检测qPCR扩增效率。

1.10 Western blot检测蛋白表达

如WANG等[15]所述, 用RIPA缓冲液辅以蛋白酶抑制剂Cocktail对其进行裂解和刮除。根据制造商提供的说明书, 使用BCA检测试剂盒测定上清蛋白浓度。用10% GFAP或12% S-100 SDS-PAGE分离蛋白质, 并将其转移到PVDF膜上。5%脱脂奶粉封膜2 h后, 用GFAP兔多克隆抗体(1:1 000)和S-100兔多克隆抗体(1:500)于4 °C孵育过夜。用TBST洗涤3次后, 用稀释为1:5 000的二抗在室温条件下孵育2 h。最后用ECL化学发光法显影拍照, 使用ImageJ进行分析。

1.11 统计学分析

数据采用SPSS 20.0软件进行分析, 定量数据以均数±标准差表示。组间差异采用单因素方差分析和Duncan's多重比较检验。$P<0.01$为极显著差异。

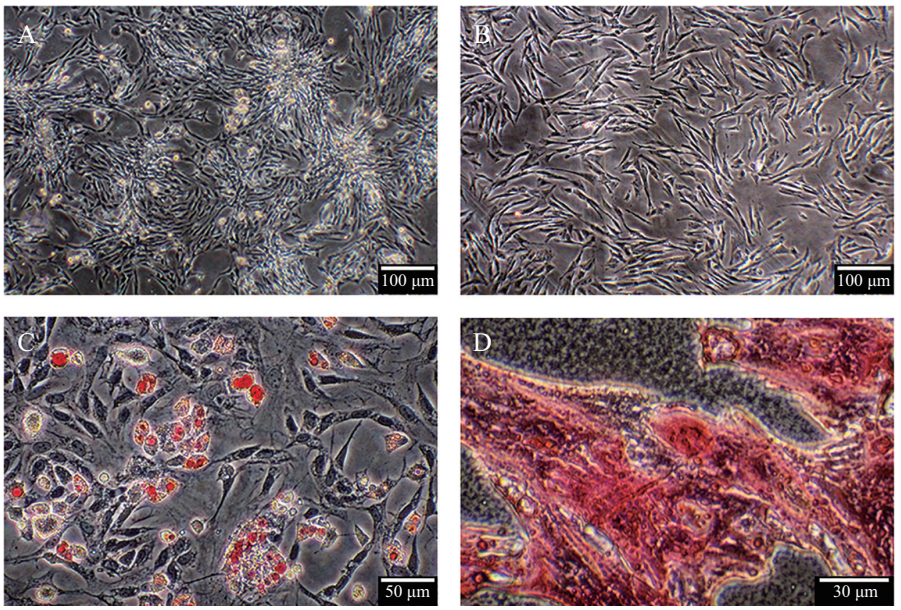
2 结果

2.1 ADSCs的培养与鉴定

原代ADSCs成团生长, 呈圆形纺锤形(图1A)。第3代ADSCs呈成纤维细胞样细胞, 漩涡状或平行生长模式(图1B)。第3代ADSCs成脂诱导后, 油红O染色可见红色脂滴(图1C)。成骨诱导后, 茜素红染色能够观察到钙化结节(图1D)。流式细胞仪表型分析显示, 细胞表面抗原CD44、CD90呈阳性(图2B和图2D), 阳性率分别为98.6%和99.2%; CD34、CD45呈阴性(图2A和图2C)。

2.2 MT对ADSCs增殖的影响

采用CCK-8法确定MT处理ADSCs的合适浓度。如图3所示, 结果表明, 与对照组相比, 50 nmol/L和100 nmol/L MT处理后, ADSCs的增殖活力较好, 两

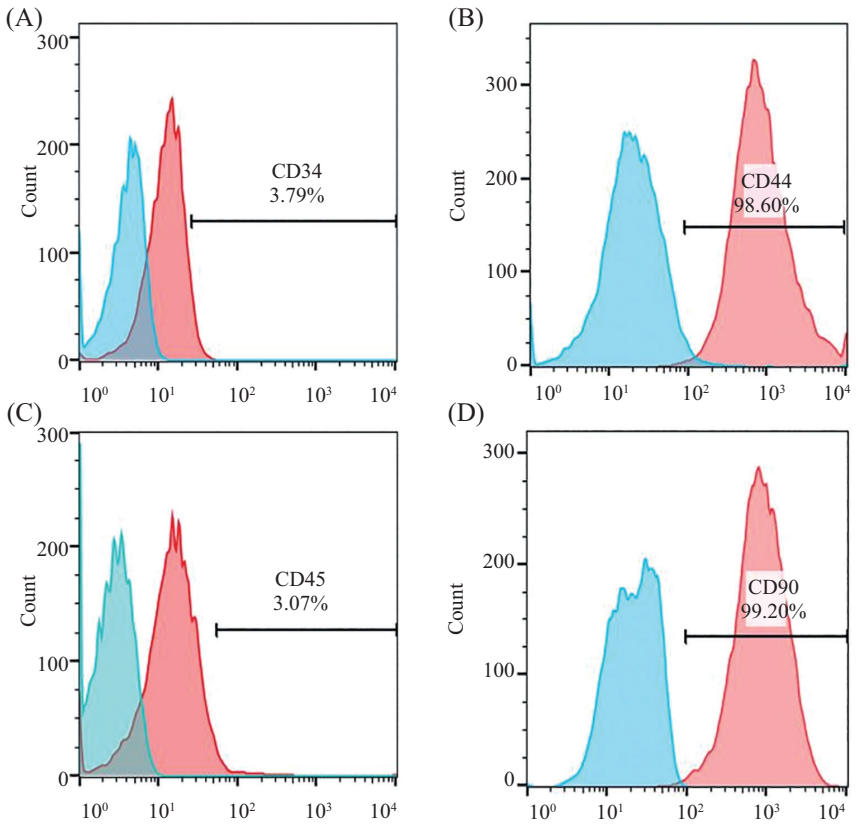


A: 原代ADSCs; B: 第三代ADSCs; C: 油红O染色; D: 茜素红染色。

A: the primary ADSCs; B: the third generation ADSCs; C: oil red O staining; D: Alizarin red staining.

图1 ADSCs的形态与多向分化

Fig.1 Morphology and multidirectional differentiation of ADSCs



A: CD34; B: CD44; C: CD45; D: CD90.

图2 流式细胞术检测ADSCs的表面标志

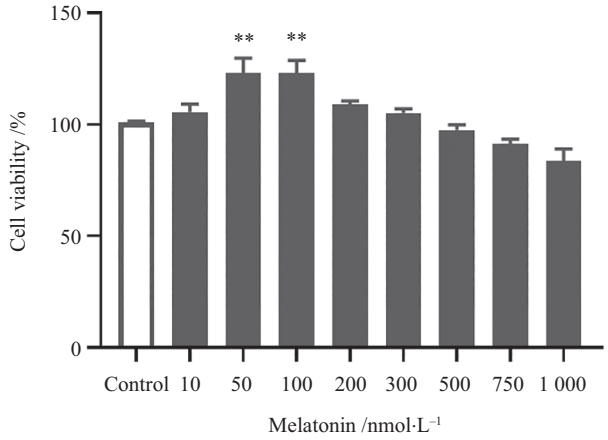
Fig.2 The surface mark of ADSCs was analyzed by flow cytometry

个浓度的MT毒性较低(P<0.01)。50 nmol/L与100 nmol/L MT对ADSCs的增殖活力影响差异不显著。所以, 本实验选用了低浓度的MT, 即50 nmol/L MT。

2.3 MT对ADSCs向SCs分化的影响

与对照组(图4A和图5A)相比, 50 nmol/L MT组的ADSCs细胞状态相对较好且细胞数量显著增加(图4B、图5B和图5E)。用神经诱导液和50 nmol/L MT+神经诱导液诱导后的细胞呈梭形生长, 可见两级突起, 相邻细胞突起之间相互连接, 呈树杈状排列(图4C和图4D)。但与神经诱导液组相比, 50 nmol/L MT+神经诱导液组诱导的S-100阳性细胞数量显著增加(图5C和图5D), 且对照组和50 nmol/L MT组无S-100的表达。

qPCR检测各组SCs标志物(*GFAP*、*S-100*)mRNA的表达情况。与对照组相比, 50 nmol/L MT组*GFAP*和*S-100* mRNA的表达水平差异不显著, 而神经诱导液组和50 nmol/L MT+神经诱导液组*GFAP*和*S-100* mRNA的表达显著上调(P<0.01), 且50 nmol/L

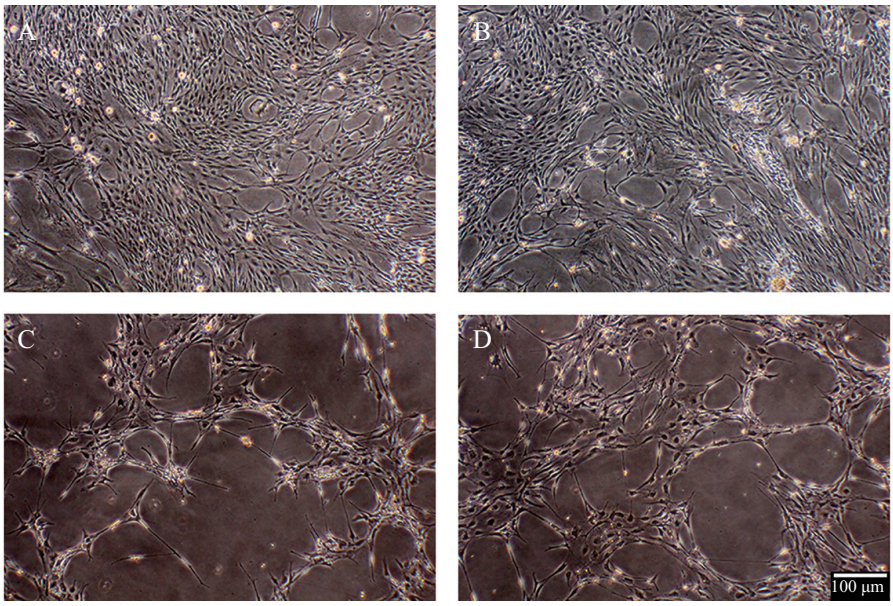


**P<0.01, 与对照组相比较。

**P<0.01 compared with control group.

图3 不同浓度MT对ADSCs增殖活力的影响

Fig.3 Effects of different concentrations of MT on proliferation activity of ADSCs

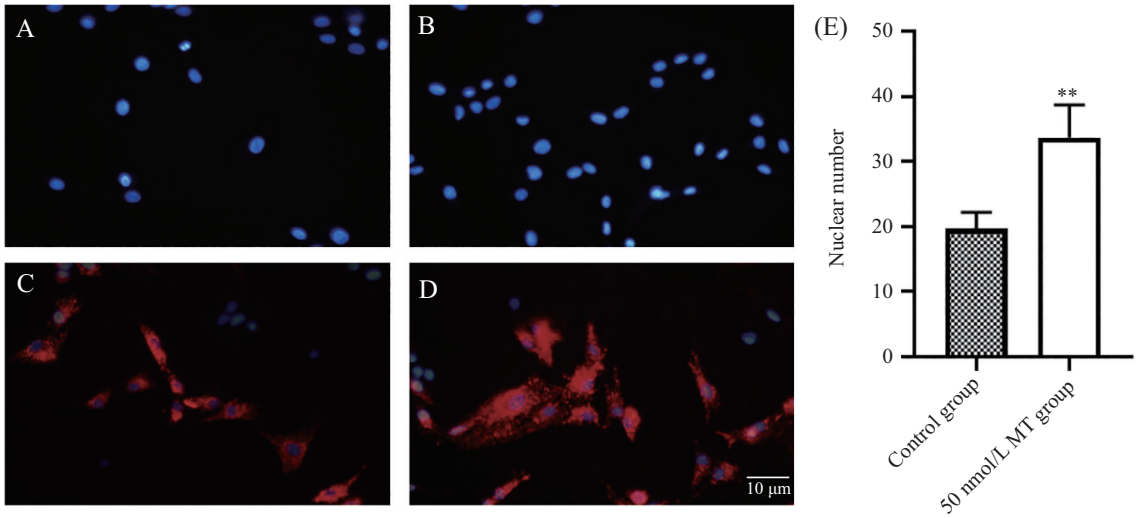


A: 对照组; B : 50 nmol/L MT组; C: 神经诱导液组; D: 50 nmol/L MT+神经诱导液组。

A: control group; B: 50 nmol/L MT group; C: neural induction fluid group; D: 50 nmol/L MT combined with nerve induction solution group.

图4 显微镜下观察诱导培养基处理第3代ADSCs的形态

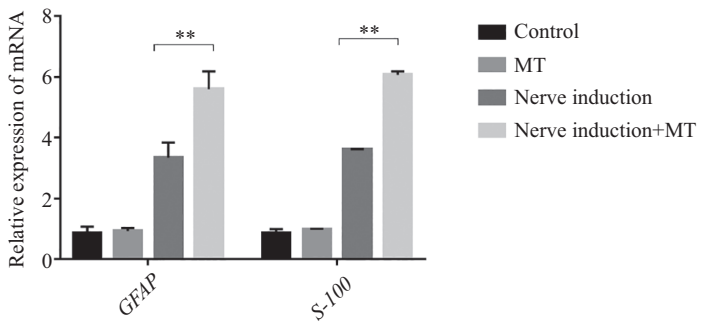
Fig.4 The morphology of ADSCs at passage 3 treated by induced medium was observed by microscope



A: 对照组; B: 50 nmol/L MT组; C: 神经诱导液组; D: 50 nmol/L MT+神经诱导液组; E: 细胞核数量统计。**P<0.01, 与对照组相比较。
A: control group; B: 50 nmol/L MT group; C: neural induction fluid group; D: 50 nmol/L MT combined with nerve induction solution group; E: nuclear count. **P<0.01 compared with the control group.

图5 免疫荧光法检测S-100蛋白的表达

Fig.5 The expression of S-100 protein was detected by immunofluorescence



**P<0.01, 与神经诱导组相比较。
**P<0.01 compared with the nerve induction group.

图6 qPCR检测*GFAP*和*S-100* mRNA的表达水平

Fig.6 The expression level of *GFAP* and *S-100* mRNA was detected by qPCR

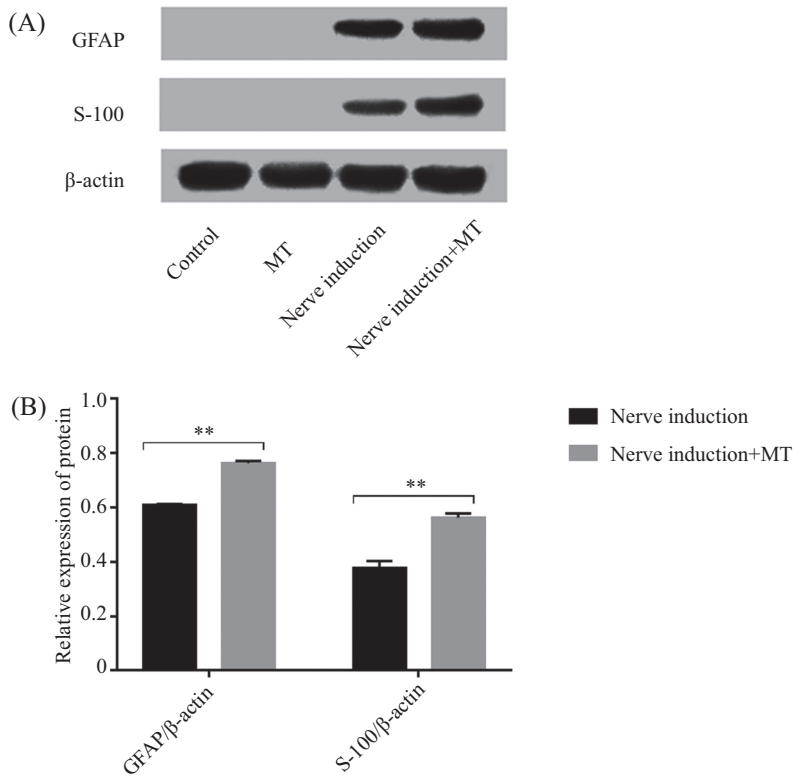
MT+神经诱导液组*GFAP*和*S-100* mRNA的表达水平更高(图6)。

Western blot检测各组SCs标志物(GFAP、S-100)蛋白的表达情况。结果显示, 对照组和50 nmol/L MT组均无GFAP、S-100蛋白的表达。神经诱导液组和50 nmol/L MT+经诱导液组均有GFAP和S-100蛋白表达(图7), 且50 nmol/L MT+神经诱导液组比神经诱导液组其表达水平显著增加。

3 讨论

SCs作为PNI的内源性修复细胞, 具有去分化、迁移、增殖、表达生长促进因子和髓鞘再生的能力, 是PNI修复中研究最广泛的细胞之一, 在PNI后的再生中起着重要作用。PNI后, SCs会转移至损伤部位, 参与神经内源性修复过程[16-17]。然而, 自体SCs很难在短时间内进行培养和增殖。因此, 需要在体外获取大量且高纯度的SCs。

ADSCs来源丰富, 易于增殖, 具有多能性, 可以分化成施万样细胞, 已被证实可以作为SCs的来源。如研究表明, 施万样细胞可以从含有多种细胞因子的ADSCs中获得[18-19]。我们之前的研究也表明, 通过添加坐骨神经渗滤液分泌的营养因子, ADSCs可以分化成施万样细胞[20]。近年来, 来自ADSCs的施万样细胞能够促进轴突生长, 有着明显促进神经再生的作用, 已被用于治疗PNI。LIU等[21]研究表明, 用神经诱导液处理ADSCs促进了轴突的长度增



**P<0.01.

图7 Western blot检测GFAP和S-100的表达水平

Fig.7 Expression level of GFAP and S-100 was detected using Western blot

长, 增强了PNI的修复作用。GEORGIOU等[22]和SEO等[23]将ADSCs分化的施万样细胞移植入大鼠体内后, 发现ADSCs分化的施万样细胞能够增加神经轴突比例, 促进了受损神经再生。此外, 还有研究比较了神经分化的ADSCs和ADSCs的治疗效果, 结果发现, 坐骨神经缺损后, 移植神经分化的ADSCs较移植ADSCs治疗效果好[24]。由此可见, ADSCs可以作为一种替代SCs治疗PNI的方法。

虽然ADSCs能够分化为SCs, 但在体内和体外的分化效率不高, 易受微环境的影响而发生炎症、凋亡和氧化应激。KINGHAM等[5]发现, 在各种胶质生长因子作用下的大鼠ADSCs中, GFAP和S-100蛋白的表达量分别为44.5%和42.9%。JIANG等[25]发现分化5天后, ADSCs分化的SCs标志物GFAP和S-100的比例分别为69.8%和72.4%。所以, 促进ADSCs增殖和分化成为了治疗PNI的关键。

MT是一种高度保守、分子量低且富含半胱氨酸的蛋白, 具有清除自由基、抗炎、抗肿瘤等多种功能[26-27]。大量研究表明, MT对细胞增殖具有促进作用。ZHANG等[28]证实, MT能够通过调控Wnt信号通路促进山羊毛囊干细胞增殖和增强其多能性。TOCHARUS等[29]和SOTTHIBUNDHU等[30]研究发现, MT能够通过作用于MT2受体, 促进3T3-L1前脂肪细胞和成年大鼠海马祖细胞的增殖。CHANG等[31]报道, MT增加了从成年小鼠脑室下区获得的神经干细胞(NSC)的数量。还有研究表明, MT能诱导SCs[32]和正常人骨细胞[33]的增殖。然而, MT对细胞的增殖也具有抑制作用。例如研究表明, MT可以在体外抑制人乳腺癌细胞[34]、脐静脉内皮细胞[35]及大鼠牙乳头细胞的增殖[36]。目前, 已知MT对细胞的增殖既有促进作用也有抑制作用。有研究表明, MT对细胞增殖的影响与使用的浓度有关[37-38]。但是, 同种浓度对不同细胞的增殖又有着不同的影响。例如, YOO等[39]发现100 μmol/L浓度的MT可以促进小鼠胚胎干细胞(ESc)的增殖。然而, MORIYA等[40]发现, 在药理学浓度(1~100 μmol/L)下, MT能够抑制表皮生长因子(EGF)刺激的NSC增殖。相同浓度(100 μmol/L)的MT对ESc和NSC的细胞增殖作用相反, 表明MT对不同类型细胞的增殖作用不同。本研究探讨了不同浓度的MT对ADSCs增殖作用的影响。结果表明, 50 nmol/L和

100 nmol/L MT能显著增强ADSCs的增殖活力, 且二者对ADSCs的增殖活力无显著性差异。

选用50 nmol/L MT处理ADSCs诱导分化后, SCs标志物GFAP和S-100 mRNA和蛋白的表达水平明显增加, 说明MT可以增加ADSCs分化成SCs的效率。本课题组先前研究显示, MT可以显著抑制ADSCs向SCs诱导分化过程中的凋亡发生[14], 表明MT促进ADSCs的增殖和向SCs分化可能与MT的抗细胞凋亡特性有关。为了进一步研究促进分化的机制, 我们还进行了体外细胞通路实验, 结果表明, MT可以通过MEK/ERK1/2信号通路促进PC12细胞的神经分化[41]。此外, 有研究表明, MT还可以通过减轻氧化应激和炎症反映来保护细胞免受损伤[42-43]。如LIU等[44]研究报道, 在IL-1β诱导的炎症环境中, MT具有维持间质干细胞存活和促进其成骨分化的能力。SHU等[45]研究发现, MT通过激活MT受体和PI3K/AKT信号通路, 显著促进诱导多能干细胞向神经细胞分化。PARK等[46]研究证实, MT通过BMP/ERK/Wnt信号通路促进MC3T3-E1细胞向成骨细胞分化和矿化。HAN等[47]研究表明, MT通过上调miR-526b-3p和miR-590-5p促进人骨髓间充质干细胞(BMSCs)向软骨细胞分化。目前, 对于MT调控ADSCs分化的细胞内信号系统调控机制现在研究的较少, 还需要进一步研究。

本研究表明, 用50 nmol/L MT处理ADSCs可以促进ADSCs的增殖, 且在神经诱导液的协同作用下, MT也可以促进ADSCs向SCs分化。这说明褪黑激素作为体内重要的神经内分泌激素, 参与促进ADSCs向神经元方向的分化成熟, 为更好地研究ADSCs向神经元细胞转化提供了一定的实验基础, 并为ADSCs联合MT治疗PNI提供理论依据。

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