N-acetylcysteine protects bone marrow stromal cells against the toxicity of 6-hydroxydopamine★

Zhang Qi-lin, Luo Wei-feng, Wang Heng-hui, Ye Yan, Zhu Ting-ge, Liu Chun-feng

Abstract

BACKGROUND: 6-hydroxydopamine, as an endogenous toxic factor in the pathogenesis of Parkinson's disease, participates in oxidative stress. N-acetylcysteine resists oxidation and removes free radicals effectively.

OBJECTIVE: To investigate the toxicity of 6-hydroxydopamine in bone marrow stromal cells and the antagonistic effect of N-acetylcysteine on it.

METHODS: Bone marrow stromal cells of Sprague-Dawley rats were cultured *in vitro*. Bone marrow stromal cells of passage 3 were treated with 6-hydroxydopamine with the final concentrations of 0, 0.05, 0.1 g/L and N-acetylcysteine with the final concentrations of 0, 0.075, 0.3, 1.2, 4.8 g/L, respectively.

RESULTS AND CONCLUSION: MTT assay showed that 6-hydroxydopamine (0.05 and 0.1 g/L) significantly decreased the viability of bone marrow stromal cells. This toxic effect of 6-hydroxydopamine was significantly inhibited by 0.3 g/L N-acetylcysteine. It suggests that antioxidant N-acetylcysteine may affect the toxic action of 6-hydroxydopamine.

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Received: 2011-07-23
Accepted: 2011-08-05
(20110614011/YJ)

Zhang QL, Luo WF, Wang HH, Ye Y, Zhu TG, Liu CF. N-acetylcysteine protects bone marrow stromal cells against the toxicity of 6-hydroxydopamine. Zhongguo Zuzhi Gongcheng Yanjiu. 2012;16(6): 985-988.

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INTRODUCTION

Bone marrow stromal cells (BMSCs) are widely researched adult stem cells, which can be differentiated into connective tissue, muscle, bone, cartilage and fat cells[1-4]. Evidence has accumulated that BMSCs of human, rats and mice can cross germ layer boundaries under certain conditions; and it can develop into neurons, even dopamine (DA) neurons[5-8]. Moreover, some studies have shown that BMSCs can release growth factors which can provide nutrition for the DA neurons, such as brain-derived neurotrophic factor, fibroblast growth factor-2 and glial cell line-derived neurotrophic factor. Furthermore, BMSCs can promote embryonic stem cells to generate DA neurons and prevent the death of DA neurons caused by 6-hydroxydopamine (6-OHDA)[8-9].

The neurotoxin 6-OHDA, commonly used to generate experimental models of Parkinson disease (PD), is thought to induce the neuropathological and biochemical characteristics of PD. However, accumulation of endogenous 6-OHDA is found in PD patients[10]. 6-OHDA shares some structural similarities with DA, exhibiting a high affinity for several catecholamine plasma membrane transporters such as the dopamine transporters (DAT). Once transported into the neurons, 6-OHDA initiates the cell death pathways by generating free radicals and inhibiting mitochondria. 6-OHDA neurotoxicity is initiated via oxidative products. It is widely reported that 6-OHDA induces neurotoxicity in a range of *in vitro* neuronal models, including rat adrenal pheochromocytoma cell line (PC 12), human neuroblastoma cell lines, primary neuronal cultures and so on[11-13]. N-acetylcysteine (NAC) is a precursor of glutathione; it is a potent antioxidant and a free radical scavenger. Its antioxidant activity protects cells from apoptosis in some pathological conditions[14-15]. In this study, we aimed to investigate the neurotoxicity of 6-OHDA in BMSCs and the protection of NAC against it.

MATERIALS AND METHODS

Design

A comparative observation on the cellular level.

Time and setting

This study was performed at the Laboratory of the Second Affiliated Hospital of Soochow University between March 2009 and September 2009.

Materials

Ten Sprague-Dawley rats weighted 60–90 g, male or female, irrespective of age were obtained from the Experimental Animal Center of Soochow University (SCXK(Su)2007-0007). Animals were housed under a 12-hour light-dark cycle with free access to food and water.

Methods

Culture of BMSCs

Sprague-Dawley rats were euthanized via cervical dislocation and then immersed in 75% ethanol for 10 minutes. The bilateral femurs, tibias and humerus were excised under sterile conditions; the connective tissues and bone ends were cut off. 20 mL marrow was extruded and placed into DMEM/F12 medium containing 10% volume fraction of fetal bovine serum (FBS); then the cells were centrifuged at 1 000 r/min for 5 minutes. The marrow cells were cultured at the concentration of 1×10^{10}/L after supernatant removal. The nonadherent cells were removed in the 72nd hour after cultivation. The medium was replaced every 3 days as the cells were grown to 80% confluence. Then the cells were digested by 0.25% trypsin and the cell concentration was adjusted to 1×10^{9}/L [16].

Intervention of 6-OHDA and NAC

The third passage cells were seeded into 96-well plates at 2×10^{8}/L. The cells were treated with

6-OHDA (final concentrations 0, 0.05 and 0.1 g/L) and NAC (final concentrations 0, 0.075, 0.3, 1.2 and 4.8 g/L) after two days.

MTT analysis of cell survival

Cells were collected from each group in 12 and 36 hours after culture, and then washed three times with PBS; cells were cultured for 4 hours after adding 2.5 g/L MTT to measure the absorbance values. The percentage of living cells was calculated and compared with that of the control group. The experiment was repeated three times.

Main outcome measures

Cell viability of BMSCs.

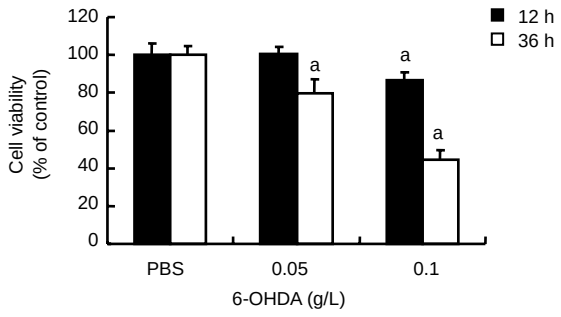
Statistical analysis

Data were expressed as mean±SD. Analysis of variance was used to assess the statistical significance between the groups. $P < 0.05$ was considered as statistically significant. The data were analyzed by SPSS15.0 software.

RESULTS

Effects of 6-OHDA on the survival of BMSCs

There was no toxicity of 0.05 g/L 6-OHDA to BMSCs in 12 hours after culture. However, the percentage of viable cells reduced to (79.81±7.29)% in 36 hours after culture ($P < 0.05$). The cell viability was significantly decreased in 12 hours and 36 hours after cultured with 0.1 g/L 6-OHDA ($P < 0.05$), (Figure 1).



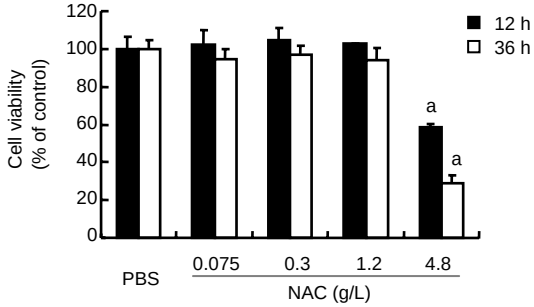
[a]$P < 0.05$, *vs.* control group

Figure 1 Viability of bone marrow stromal cells in 12 hours and 36 hours after treated with 6-hydroxydopamine (0.05 g/L and 0.1g/L) by MTT assay

Effects of NAC on BMSCs viability induced by 6-OHDA

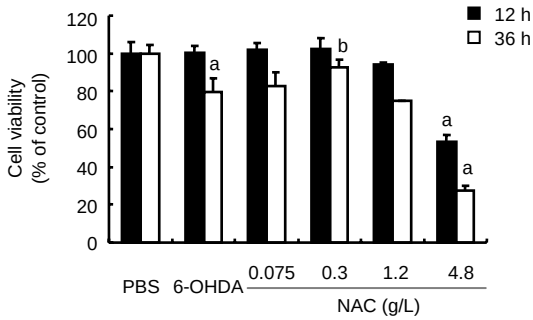
According to the results of MTT assay, NAC (0.075, 0.3 and 1.2 g/L) did not effect the cell viability of BMSCs. But NAC at the concentration of 4.8 g/L significantly reduced the survival of BMSCs (Figure 2).

To examine the effects of NAC on BMSCs viability induced by 6-OHDA, cells were incubated with 6-OHDA and NAC, as shown in Figure 3 and 4. NAC (0.3 g/L) attenuated the toxicity of 6-OHDA at the concentrations from 0.05 to 0.1 g/L in BMSCs. BMSCs viability induced by 0.1 g/L 6-OHDA was increased in 12 hour after cultured with NAC at the concentration of 1.2 g/L ($P < 0.05$). It indicates that NAC has a protective effect on BMSCs intervened with 6-OHDA.



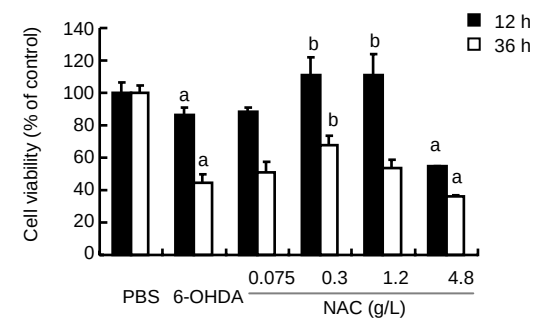
[a]$P < 0.05$, *vs.* control group

Figure 2 Viability of bone marrow stromal cells in 12 hours and 36 hours after treated with different concentrations of N-acetylcysteine (NAC) by MTT assay



[a]$P < 0.05$, *vs.* control group, [b]$P < 0.05$, *vs.* 6-OHDA treated group

Figure 3 Viability of bone marrow stromal cells incubated with 6-hydroxydopamine (6-OHDA) (0.05 g/L) and N-acetylcysteine (NAC; different concentrations) by MTT assay



[a]$P < 0.05$, *vs.* control group, [b]$P < 0.05$, *vs.* 6-OHDA treated group

Figure 4 Viability of bone marrow stromal cells incubated with 6-hydroxydopamine (6-OHDA) (0.1 g/L) and N-acetylcysteine (NAC; different concentrations) by MTT assay

DISCUSSION

Parkinson's disease (PD) is a progressive neurodegenerative disease. Although the etiology of PD remains unknown, the patients often experience an increase in oxidative stress and a decrease in endogenous antioxidants, especially in the older people. Oxidative stress induced by the auto-oxidation of dopamine can damage cellular proteins, lipids and DNA and activate monoamine oxidase[12]. In addition,

mitochondrial dysfunction is involved in the pathogenesis of PD[17-18]. However, the specific pathogenesis remains unclear. So far, the most effective treatment for PD is Levodopa[19-20]. Cell therapy is an effective therapeutic tool for PD[21]. BMSCs may serve as a cell source for the treatment of PD because it is readily available with no with ethical problems. Studies have shown that the function of dopaminergic systems in the substantia nigra and striatum was restored by BMSCs transplantation, and the behavior was improved in the animal models of PD[22-24].

6-OHDA is known as a dopaminergic neurotoxin and has been widely applied in a range of *in vitro* models[25-26]. However, the toxicity of 6-OHDA in BMSCs is not well defined. Our study shows that 6-OHDA-induced toxicity in BMSCs is concentration and time dependent. Oxidative stress may play an important role in this process. 6-OHDA induces neurotoxicity via p-benzoquinone and hydrogen peroxide generated from auto-oxidation[13, 27-28]. To determine whether 6-OHDA-induced toxicity in BMSCs is initiated *via* oxidative stress, the experiments were conducted in the presence of the anti-oxidants NAC.

NAC is the precursor of glutathione (GSH), which can reduce cystine or act as an intracellular source of sulfhydryl groups [29]. As an antioxidant, NAC participates in the protection of cytotoxic cells under some pathological conditions[30]; studies have shown that NAC has a neuroprotective effect on neurodegeneration disease and PD[31]. Cell death induced by 6-OHDA is prevented by preincubation with NAC for 30 minutes before 6-OHDA treatment[13, 28]. However, it is ineffective when NAC is added immediately after 15 minutes exposure to 6-OHDA[13]. In our study, NAC at the concentrations of 0.075 −1.2 g/L is safe for BMSCs. NAC with the concentration of 4.8 g/L has a significant influence on BMSCs. However, the possible mechanism is unclear. In the meantime, 0.3 g/L NAC can significantly reduce the toxicity of 6-OHDA in BMSCs.

Based on these findings, we recommend PD patients use NAC to reduce the toxic effect of 6-OHDA on BMSCs.

ACKNOWLEDGMENTS: We would like to express our thanks to Zhan Zhi-lin from the Second Affiliated Hospital of Soochow University, who gave us considerable direction and help in this study.

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N-乙酰半胱氨酸拮抗 6-羟基多巴胺对骨髓基质细胞的毒性作用*

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摘要

背景：内源性 6-羟基多巴胺能参与氧化应激，N-乙酰半胱氨酸能有效抗氧化和清除自由基。

目的：探讨 6-羟基多巴胺对骨髓基质细胞的毒性作用及 N-乙酰半胱氨酸对其的拮抗作用。

方法：在体外培养 SD 大鼠骨髓基质细胞，取第 3 代骨髓基质细胞分别加入终浓度为 0，0.05，0.1 g/L 的 6-羟基多巴胺和终浓度为 0，0.075，0.3，1.2，4.8 g/L 的 N-乙酰半胱氨酸。

结果与结论：MTT 检测发现 0.05 和 0.1 g/L 6-羟基多巴胺可以明显降低骨髓基质细胞的细胞活性，而加入 6-羟基多巴胺的同时加入 0.3 g/L N-乙酰半胱氨酸可以显著抑制 6-羟基多巴胺的毒性作用。提示抗氧化剂 N-乙酰半胱氨酸可以影响 6-羟基多巴胺的作用。

关键词：毒性；6-羟基多巴胺；N-乙酰半胱氨酸；骨髓基质细胞；大鼠；MTT
doi:10.3969/j.issn.1673-8225.2012.06.008
中图分类号: R394.2　文献标识码: A
文章编号: 1673-8225(2012)06-00985-04
张琪林，罗蔚锋，王恒会，叶艳，朱婷鸽，刘春风. N-乙酰半胱氨酸拮抗 6-羟基多巴胺对骨髓基质细胞的毒性作用[J].中国组织工程研究，2012，16(6):985-988.
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(Edited by Li YK, Li X/Wang L)

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本文创新性：国内外有较多研究对骨髓基质细胞移植治疗帕金森病及 6-羟基多巴胺对多种细胞，如 PC12 细胞、多巴胺能神经元及 SH-SY5Y 细胞的损伤作用及可能机制进行探讨，但是对 6-羟基多巴胺这种内源性的神经毒性物质对骨髓基质细胞的研究较少，本实验就此进行研究。

展示中国组织工程研究最优秀的成果 ②

(接目次页)

虽然一些研究已经显示干细胞可以形成各种类型的细胞和简单的组织，但是否已具有可形成多种复杂器官的能力目前还远未清楚。来源于干细胞的细胞应用于细胞和组织替代治疗后仍面临着移植排斥的问题 ，用于临床治疗的安全性问题。对于胚胎干细胞而言，在移植前如何真正保证胚胎干细胞全部被诱导分化，对诱导分化的细胞又怎样才能做到严格纯化 ，如何认识干细胞可塑性的机制，干细胞分化时所处微环境中的调控因素又是如何起作用的，等等问题，都需要去认识去实验去观察呀……

探讨组织工程研究中支架材料的构建：支架材料构建的基本原则，支架材料的应用策略，常用支架材料的种类和来源，天然可降解的生物材料，人工合成可降解的生物材料，新型生物材料，常用天然支架材料及其制备，包括胶原的制备，脱细胞真皮的制备，脱细胞周围神经组织的制备，脱细胞骨组织的制备等。具备良好的组织相容性、良好的表面活性、可塑性、生物可降解性、具有三维立体结构的理想的组织工程的基质材料又有哪些？

组织工程研究中种子细胞与支架材料将怎样进行联合培养？ 联合培养中的方法与技术有哪些新突破?细胞接种前的准备，细胞与支架的接种技术，细胞示踪技术中的病理学示踪和非病理学示踪……

建立由细胞和生物材料构成的三维空间复合体，这与传统的细胞培养二维结构本质上的区别就在于其最大优点是可形成具有生命力的活体组织，对病损组织进行形态、结构和功能的重建并达到永久性替代。用最少的组织细胞通过体外培养扩增后，进行大块组织缺损的修复，按组织器官缺损的情况任意塑形，达到完美的形态修复。

在组织工程研究中不可或缺的生物反应器，其原理、技术与应用又有哪些新的进展？生物反应器的种类，生物反应器的应用技术还存在哪些问题？

探讨组织工程研究中的生长因子：常用的生长因子有转化生长因子-β，骨形态发生蛋白，碱性成纤维细胞生长因子，表皮生长因子，胰岛素样生长因子，血管内皮细胞生长因子，神经胶质生长因子，软骨调节素，血小板衍化生长因子，角朊细胞生长因子……

生长因子中的控制释放技术？携带生长因子的控释材料？生长因子的控制释放体系？生长因子控制释放体系中存在着的诸多问题也需要解决。

在获得理想细胞之前，组织构建需用自体细胞与材料复合，构建组织工程的初级产品，在这一过程中面临的最大问题是细胞增殖缓慢。解决这一问题目前有两条途径，即具有分化潜能的细胞的诱导和促生长因子的应用。在体内情况下，多种生长因子都由机体协调作用，因而是一个多因子的序贯作用体系，体外如何模仿实施这一多因子序贯调节过程也是组织工程研究中的一个重大课题。

探讨组织工程化组织和器官的构建：组织工程化肝的构建，组织工程化肾的构建，组织工程化血管和心脏瓣膜的构建，组织工程化周围神经的构建，组织工程化骨与软骨的构建，组织工程化牙齿的构建，组织工程化椎间盘的构建，组织工程化皮肤的构建，组织工程化角膜的构建，组织工程化乳腺的构建，组织工程化气管的构建，组织工程化食管的构建，组织工程化膀胱的构建，组织工程化阴茎的构建，组织工程化尿道的构建，其他更多组织工程化器官的构建？

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