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Effect of Nitric Oxide on the Expression of Transferrin Receptor in Rats with Anemia in Chronic Disease

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Abstract: Objective To study the influence of nitric oxide (NO) in rats with anemia in chronic disease (ACD) and the effect of NO on the expression of transferrin receptor (TfR) in bone marrows and to provide experimental evidence for the prevention and treatment of ACD. **Methods** The conventional animal model of rheumatoid arthritic (RA) was established by injection of Freund's complete adjuvant. On the basis of this model, we injected Freund's complete adjuvant repeatedly to establish the ACD model. The rats were randomly assigned into three groups (Group A: control group; Group B: inflammatory group; Group C: inflammatory + NO inhibitory agent group). The histopathological changes of the toe joints of the rats were observed and the contents of NO, Hb and nitric oxide synthetase (NOS), and the expression of TfR were measured in the three groups. **Results** In Group B, the contents of NO and NOS in the serum were higher than those in Group A; TfR expression in bone marrow cells was lower than that in Group A, and anemia was more severe than that in Group A. After administrating NOS inhibitory agent (L-NAME), anemia was improved; the contents of NO and NOS remarkably decreased compared with those in Group B, but were still higher than those in Group A; TfR in bone marrow cells obviously increased compared with that in Group B, but was still lower than that in Group A. **Conclusions** NO may play an important role in the pathogenesis of ACD and regulation of TfR on ACD, thereby providing experimental evidence for further study of the pathogenesis of ACD. It is helpful in hindering the development of anemia by reducing the NO level as early as possible, and is a new way of treating ACD.

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Key words: Nitric oxide; Chronic disease; Anemia; Transferrin receptor; Rat

NO对慢性病贫血大鼠转铁蛋白受体表达的影响

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[摘 要] 目的 探讨一氧化氮(NO)在慢性病贫血(ACD)发病中的作用及对骨髓血细胞转铁蛋白受体(TfR)的影响,为ACD的防治提供实验依据。**方法** 用福氏完全佐剂建立传统的类风湿性关节炎大鼠动物模型,在此基础上通过反复注射福氏完全佐剂,建立ACD大鼠动物模型。利用此模型观察对照组、炎症组及炎症+一氧化氮合酶(NOS)抑制剂组的NO浓度的改变、贫血的程度及与TfR的关系。**结果** 炎症组NO、NOS浓度显著高于对照组,贫血明显,TfR表达强度低于对照组,差异有显著性($P<0.01$);用NOS抑制剂后,NO和NOS水平低于炎症组但仍高于对照组,贫血改善,TfR介于炎症组与对照组之间,差异有显著性($P<0.01$)。**结论** NO参与了ACD的发病及ACD时TfR的调节,为从NO对TfR影响角度进一步认识ACD的发病机制提供了实验依据;及早降低NO水平,有利于阻止贫血的发展,为ACD的治疗提供一条新途径。

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[关 键 词] 一氧化氮;慢性病;贫血;转铁蛋白受体;大鼠

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At present pathogenesis of anemia in chronic disease (ACD) is unclear. The effect of nitric oxide (NO) on the pathogenesis of ACD has been emphasized recently. In this article we investigated the relationship between the NO level and the transferrin receptor (TfR) in bone marrow cells in different treated groups by establishing the model of ACD in rats to explore the mechanism of ACD and provide experimental evidence for the prevention and treatment of ACD.

Materials and methods

Reagents

Freund's incomplete adjuvant and nitric oxide synthetase inhibitory agent N^G-nitro-L-arginine methl ester (L-NAME) were obtained from Sigma US. Bacille Calmette Guerin was obtained from Chengdu Biological Institute. Purified anti-rat CD_{71} (transferrin receptor) was obtained from Pharminge US. The biotin-streptavidin-peroxidase kit was prepared by Beijing Yitai Company. The NO kit was obtained from Jingmei Biotechnological Company. The NOS kit was purchased from Nanjing Biotechnological Institute.

Experimental animals

Twenty 6 to 7-week-old male SD rats (body weight 210－240 g) were obtained from the Experimental Animal Center of Sichuan University.

Model of rat ACD

Bacille Calmette Guerin was inactivated for one hour at 80℃, and it was then added into Freund's incomplete adjuvant (final concentration was 10 mg/ml) to make Freund's complete adjuvant (FCA). The rats were randomly assigned into three groups (10 rats in each group). The rats in the control group (Group A) were injected with 100 μl normal saline (NS) in their right hindfoot. The rats in the inflammatory group (Group B) and the inflammatory + inhibitory agent group (Group C) were respectively injected with 100 μl Freund's complete adjuvant in the corresponding sites of Group A. One month later, the rats in Group B and C were injected with Freund's complete adjuvant twice within an interval of ten days, and Group A rats were injected with NS twice instead of FCA. Meanwhile Group C rats were fed with water supplement with L-NAME (1 mg/ml) and had an weekly injection of L-NAME (15 mg/kg).

Measurement of the volume of rats' feet

By the method of water volume[1], the volume of each rat hindfoot was measured before, and 14 days, 30 days and 60 days after injection of FCA.

Preparation of the tissue section of rats' feet

On the 60th day after injection of FCA, the rats were sacrificed. The digits of the left hindfoot were fixed in 15% formaldehyde, and then dehydrated by alcohol and coated by wax. After being sectioned, the sections were stained with HE.

Hemoglobin (Hb) assay

The Hb content was measured by obtaining 300 μl of tail blood before and 30 and 60 days after injection of FCA.

NO and NOS assay

On the 60th day after injection of FCA, the contents of NO and NOS of the blood (4 ml blood obtained from the rat heart) were detected according to the instruction provided by the literature that came with the kits.

Assay of TfR expression in bone marrow cells

After 60 days of FCA injection, the rats were sacrificed. The femurs were cut and flushed with 0.5 ml ice-cold RPMI 1640, and then Ficool was added into them. After centrifugation for 20 minutes at 1800-2000 rPm, the separated mononuclear cells were washed three times by 1640. The glass slides were smeared with cell suspension (1×10^6/L) and were left to dry. After the mouse anti-rat CD_{71} (2.5 μg/ml) was added, the slides were put into a moist box for 30 minutes at 37℃ and sheep anti-mouse IgG labeled with biotin was added. Under an oil microscope, the positive cells of different degrees among the 200 monocytes were counted and the positive index was calculated by counting the number of positive cells of different degrees among 100 cells. The strong positive cells (+ + +) carry a score of 3; standard positive cells (+ +) 2; weak positive cells (+) 1; negative cells (－) 0. The total scores of positive cells of different degrees stand for the positive index.

Statistical analysis

The data were analyzed by the SPSS 10.0 software according to the one-way analysis of variance and Student-Newman-keuls multiple range test.

Results

Changes of the volume of rat hindfeet in each stage

The difference of the volume of hindfeet before injection of FCA among the three groups was not significant ($P > 0.05$). On the 14th, 30th and 60th day after injection of FCA, the difference of the volume of hindfeet among the 3 groups was significant ($P < 0.01$). And the volumes of left and right hindfeet in Group B were bigger than those in Group A ($P < 0.01$), while those in Group C were smaller than those in Group B, but still bigger than those in group A ($P < 0.01$) (See Table 1).

Table 1 Changes of the volume of hindfeet in each group ($n = 10$, $\bar{x} \pm s$, cm^3)

Groups	0 d		14 d		30 d		60 d	
	left	right	left	right	left	right	left	right
A	1.15±0.11	1.14±0.05	1.34±0.05	1.33±0.08	1.60±0.06	1.64±0.06	2.05±0.05	2.08±0.08
B	1.14±0.02	1.15±0.11	1.86±0.14[a]	2.38±0.35[a]	2.32±0.37[a]	2.65±0.18[a]	3.40±0.20[a]	3.74±0.15[a]
C	1.15±0.01	1.15±0.02	1.52±0.26[a,b]	1.91±0.37[a,b]	1.83±0.14[a,b]	1.10±0.11[a,b]	2.98±0.10[a,b]	3.07±0.14[a,b]
F	0.07	0.06	20.98	28.10	22.81	143.74	34.77	275.25
P	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: a vs Group A $P < 0.01$; b vs Group B $P < 0.01$

Histopathological changes of the left toe joint of the hindfeet in each group

In Group A, the joint cartilage was not damaged and synovial membrane cells did not proliferate and were not infiltrated by inflammatory cells (See Figure 1). In Group B, synovial membrane cells proliferated notably, and a lot of plasmocytes and lymphocytes were infiltrated in the villi of the synovial membrane. The stromata of the cartilage was dissolved (See Figure 2). In Group C, synovial membrane cells proliferated slightly. The infiltration of inflammatory cells decreased obviously. The damage of the stromata of the cartilage was not remarkable (See Figure 3).

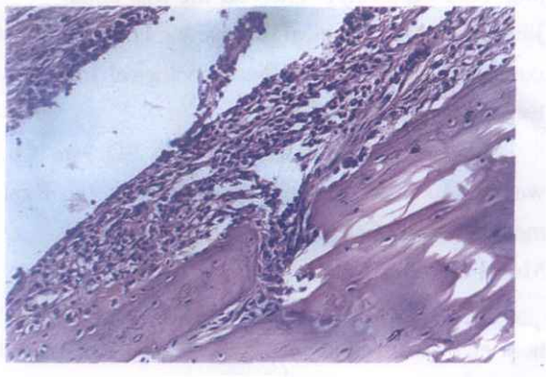

Figure 2 Histopathological changes of the left toe joint of the hindfeet in Group B

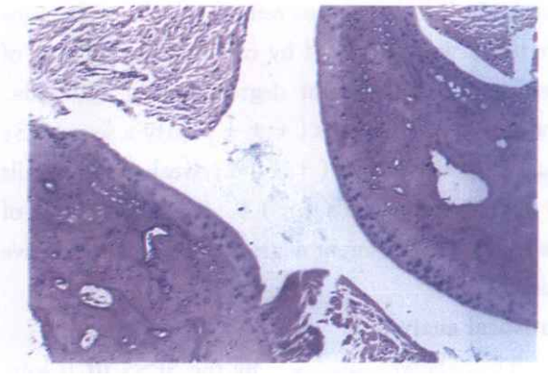

Figure 1 Changes of the left toe joint of the hindfeet in Group A

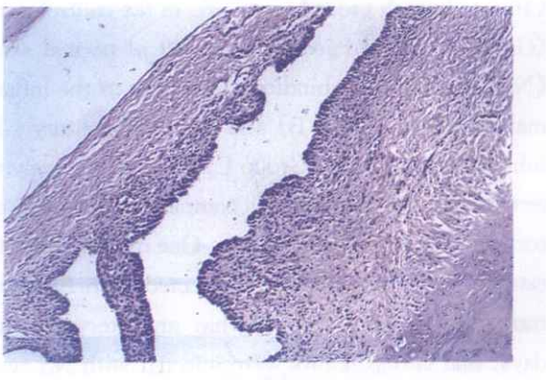

Figure 3 Histopathological changes of the left toe joint of the hindfeet in Group C

Contents of Hb in each group

The differences of the Hb content before and after 30 days of FCA iniection among the three groups were not significant ($P > 0.05$). On the 60th day after FCA administration, notable anemia appeared in Group B ($P < 0.01$). But anemia was improved in Group C, although it was still more severe than that in Group A ($P < 0.01$ or 0.05) (See Table 2).

Table 2 Changes of the Hb contents in each group (n=10, $\bar{x} \pm s$, g/L)

Group	0 d	30 d	60 d
A	155.60±5.13	156.50±8.21	158.40±4.37
B	155.30±6.48	154.90±5.32	134.10±8.15[a]
C	154.70±4.21	153.30±2.54	149.20±7.35[b,c]
F	0.06	2.70	29.13
P	>0.05	>0.05	<0.01

Note: a vs Group A $P < 0.01$; b vs Group B $P < 0.01$; c vs Group A $P < 0.05$

Contents of NO and NOS in each group

In Group B, the contents of NO and NOS were higher than those in Group A. In Group C, after L-NAME administration, they decreased remarkably compared with those in Group B, but were still higher than those in Group A ($P < 0.01$) (See Table 3).

Table 3 Contents of NO and NOS in each group (n=10, $\bar{x} \pm s$)

Groups	NO (μmol/L)	NOS (U/ml)
A	36.20±3.56	9.59±1.37
B	79.50±6.17[a]	26.24±4.61[a]
C	57.10±3.34[a,b]	15.64±2.07[a,b]
F	226.49	216.66
P	<0.01	<0.01

Note: a vs Group A $P < 0.01$; b vs Group B $P < 0.01$

Changes of the positive index of TfR in the bone marrow

In Group B, the positive indexes of + + +, + +, + were all lower than those in Group A ($P < 0.01$). In Group C, they increased compared with those in Group B ($P < 0.01$), but were still lower than those in Group A ($P < 0.01$) (See Table 4).

Table 4 Changes of the positive indexes of tranferrin receptor in each group (n=10, $\bar{x} \pm s$)

Groups	strong positive (+ + +)	standard positive (+ +)	weak positive (+)
A	64.80±9.30	59.20±8.39	20.80±2.69
B	14.70±2.98[a]	19.60±3.65[a]	34.30±3.16[a]
C	36.60±5.06[a,b]	35.60±2.95[a,b]	29.00±1.82[a,b]
F	156.53	135.57	67.26
P	<0.01	<0.01	<0.01

Note: a vs Group A $P < 0.01$; b vs Group B $P < 0.01$

Discussion

ACD is a syndrome referring to chronic infectious, inflammatory, and neoplastic disorders. The model of rat polyarthritic by injection of FCA is a classical model to study rheumatoid arthritic, the joint histopathology and blood components of which are similar to those in human rheumatoid arthritic[2]. Anemia is one of the most common symptoms except the changes of joints in rheumatoid arthritic[3]. Our study showed that the rats in Group B had obvious symptoms of polyarthritic 14 days after injection of FCA, and showed characteristics of histopathology 60 days after FCA injection. The anemia became worse when the inflammation worsened. It suggested that the decline of Hb level corresponded with the activity of rheumatoid. This model confirmed the characteristics of ACD and the findings were able to provide useful reference for clinicians.

TfR is a transmembrane protein which can bind specifically to transferrin, and iron is transferred into cells through the cell membrane by endocytosis complex of transferrin and TfR. The membranes of erythroblasts in bone marrow cells are rich in TfR expression, which accounts for 80% of total TfR. Therefore erythroblasts in the bone marrow are the major source of TfR[4]. Recent studies have demonstrated that NO plays an important role in regulating TfR expression in ACD.

TfR expression is mediated by post-transcriptional regulation[5]. Two cytoplastic RNA binding proteins, iron regulatory protein-1 (IRP-1) and IRP-2, interact with specific mRNA called iron-responsive element (IRE) to regulate the expression of TfR.

Comparisons of amino acid sequences revealed striking homology between human IRP-1 and human mitochondrial aconitase[6]. Because mitochondrial aconitase is the main target enzyme of NO, NO also regulates the activity of IRP-1. NO activates the ability of IRP-1 RNA-binding by two possible mechanisms: the direct effect on the [4Fe-4S] cluster leads to the change of IRP-1 configuration; iron mobilized from cells results in iron depletion, which increases the RNA-binding activity of IRP-1 and reduces the aconitase activity of IRP-1[7]. As a result, it leads to reduced translation of ferritin mRNA and increased stability of TfR mRNA. However, it shows contradiction with the changes of TfR in ACD.

However, some experts explained the contradiction according to the different redox states of NO. Kim found that NO could disrupt the Fe-S center of IRP-1, make the configuration of IRP-1 change, activate the IRE-binding ability of IRP-1 and increase the stability of TfR. In contrast, IRP-1 can be nitrosylated by nitrosonium ions (NO^+), which block the combination of IRE and IRP-1 and facilitate TfR degradation. Recent studies have demonstrated that IRP-2 is a very important target molecule of NO[8,9]. NO can make the thiol group of IRP-2 nitrosylated and cause inactivity and degradation of IRP-2. At the same time, the level of TfR mRNA decreases remarkably. In addition, this has taken place before the IRE-binding activity of IRP-1 is reduced by NO^+. It shows that IRP-2 alone plays a role in mediating the level of TfR mRNA.

Our study showed that TfR expression in Group B decreased obviously. After L-NAME administration, TfR expression increased remarkably. This indicated that NO played an important role in the regulation of TfR on ACD. It provided experimental evidence for further study of the pathogenesis of ACD, and provided a new way to treat ACD.

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