

Effect of Lipopolysaccharide and 4-(Methylnitro-samino)-1- (3-pyridyl)-1-butanone on the Proliferation of Mouse Bone Marrow Stem Cells

Yu Zhongjie¹, Jiang Shasha¹, Wang Yi¹, Liu Xiaoping¹✍️, Yang
Tongxi¹, Wang Chunxue¹

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¹Department of Histology and Embryology, Medical College of Qingdao University,
Qingdao266021, China

Abstract BMSCs were isolated, cultured and proliferated in vitro. The proliferation of mouse BMSCs treated with different concentrations of LPS and NNK were detected by MTT assay, the expression of NF- κ B and CUGBP1 in the BMSCs were analyzed by Immunocytochemistry assay and Western Blot. MTT results shown that different concentrations of LPS and NNK could promote the proliferation of BMSCs and the promotion effect of LPS12.5 μ g/ml, NNK10 μ g/ml and LPS12.5 μ g/ml combined NNK 10 μ g/ml was much more significant than others. Immunocytochemistry and Western Blot revealed that the location and the expression level of NF- κ B and CUGBP1 in BMSCs treated with LPS and NNK were changed with different incubation time. The highest expression of NF- κ B was detected at the LPS48h, NNK24h and LPS combined NNK24h and significant difference can be seen in-group compared. The highest expression of CUGBP1 was LPS96h, NNK48h and LPS combined NNK24h and significant difference can be seen in-group compared. The irritation of LPS and NNK on the proliferation of mouse BMSCs may mediate by up-regulating the expression of NF- κ B and CUGBP1.

Keywords Bone Marrow Stem Cells (BMSCs); Cell proliferation; NF- κ B; CUGBP1.

Introduction

Stem cells is the most primitive, pluripotent and self-renewing cells in tissue and has the ability to differentiate into osteoblast, chondroblast, adipocyte, neurocyte, cardiomyocytes, hepatocyte, with different inductions, this can be applied to therapy of various

organs damage and become the hot research of life science and medicine ^[1].

LPS, the components of gram-negative bacteria cytoderm, was considered to play an important role in gram-negative bacterial infection and disease evolution. Meanwhile, LPS is the main causes of systemic inflammatory response syndrome (SIRS) ^[2]. NNK is

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Liu Xiaoping (Correspondence)



xplyu2008@126.com



+86-150-92241129(0532-82991708)

one of the most harmful components of cigarette smoke and can cause a variety abnormal change of immune function. It can result in the production of toxins that bind DNA to form adducts that causes the mutation of oncogene and anti-oncogenewhen it is metabolized in body. One study suggests that NNK can bind with acetylcholine receptors to promote cell proliferation, survival and migration [3].

Nuclear transcription factor (NF- κ B) has the function of transcriptional activation, occurrence of inflammation, anti-apoptosis, directly involving in cell cycle regulation, and the activation of NF- κ B can promote cell proliferation [4~5], suppresses NF- κ B activation causing reduced proliferation and induction of apoptosis in cell culture [6~7]. CUG linking protein1 (CUGBP1) is a kind of RNA-binding protein that acts in the nucleus and cytoplasm to regulate alternative splicing, deadenylation, mRNA stability and translation [8]. CUGBP1 expression is associated with the proliferation of cells and its down-regulated expression inhibits cell proliferation [9]. Previous studies showed that LPS combined NNK can significantly increase the incidence of lung cancer in vivo and stimulate the proliferation of cancer cells [10]. However, the effect of LPS and NNK on the proliferation and NF- κ B, CUGBP1 protein expression of stem cellswere not clear. In this study the effect of different concentrations of LPS, NNK and LPS combined NNK on the proliferation and the expression of NF- κ B and CUGBP1 of mouse BMSCs were detected to reveal the possible mechanism of BMSCs proliferation stimulated by inflammatory.

1 Material and Method

1.1 Animal

4-week-old Kunming male mouse, weighting 25-28g, ordered from the Animal Center of Qingdao University Medical College.

1.2 The isolation, purification and amplification of mouse BMSCs

After sacrificed, the femur and tibia ofmouse were separated in clean bench and the cells in bone marrow cavities were rinsed by DMEM-high glucose medium containing 10% FBS, the cell suspension was transferred into 25ml flask and cultured in incubator with 37°C, 5%CO₂, and saturated humidity.

BMSCs were purified by differential adhesion method. The morphology of BMSCs was observed with inverted microscope. When the confluence of BMSCs reached 80%~90%, passage culture for the expansion of BMSCs.

1.3 The effect of LPS, NNK and LPS combined NNK on the proliferation of mouse BMSCs (MTT assay)

The third passage BMSCs were inoculated into 96-well plates, 1.5x10⁴ cells/well, incubated with different concentrations of LPS, NNK, LPS combined NNK and equal volumes serum-free medium (control group) respectively, 5 multiple wells in each group. The cells were cultured in incubator with 37°C, 5%CO₂ and saturated humidity for 96h (Table1). Then cells were incubated with medium containing MTT (5mg/ml) for another 4h. Supernatant was replaced by dimethyl sulfoxide, after shaking 10min, the absorbance were measured by enzyme-labeled instrument under 490nm wave length.

Table1. The Concentration of LPS, NNK and LPS combined NNK in Different Groups

Group	Concentration
Control	0µg/ml
LPS12.5	LPS12.5µg/ml
LPS25	LPS25µg/ml
LPS50	LPS50µg/ml
NNK5	NNK5µg/ml
NNK10	NNK10µg/ml
NNK20	NNK20µg/ml
LPS12.5 combined NNK5	LPS12.5µg/ml, NNK5µg/ml
LPS12.5 combined NNK10	LPS12.5µg ml, NNK10µg/ml
LPS12.5 combined NNK20	LPS12.5µg/ml, NNK20µg/ml
LPS25 combined NNK5	LPS25µg/ml, NNK5µg/ml
LPS25 combined NNK10	LPS25µg/ml, NNK10µg/ml
LPS25 combined NNK20	LPS25µg/ml, NNK20µg/ml
LPS50 combined NNK5	LPS50µg/ml, NNK5µg/ml
LPS50 combined NNK10	LPS50µg/ml, NNK10µg/ml
LPS50 combined NNK20	LPS50µg/ml, NNK20µg/ml

1.4 The expression features of NF-κB and CUGBP1 in BMSCs detected by Immunocytochemistry assay

The concentration of LPS, NNK and LPS combined NNK we used were based on the result of MTT assay. BMSCs were incubated with the medium containing LPS12.5µg/ml, NNK10µg/ml, LPS12.5µg/ml combined NNK10µg/ml respectively for 24h, 48h, 96h, then the cells were fixed by cold acetone for 30min, followed by permeabilized with 0.5% TritonX-100 for 20min at room temperature, rinsed several times with PBS, treated with 3% H₂O₂ for 30min to inactivate the endogenous enzyme, incubated with primary antibody (Rabbit anti Mouse NF-κB p65 polyclonal antibody, Rabbit anti Mouse CUGBP1 polyclonal antibody) in a humidified chamber overnight at 4°C, incubated with secondary antibody (Goat anti Rabbit HRP conjugated) for 2h at 37°C, then colorated with DAB in dark for 6min. After each treatment the cells were rinsed several times with PBS. The cells were dehydrated in gradient alcohol after rinsed with distilled water, then sealed pieces with neutral gum. Under high magnification the cells were observed, photographed and 400 cells in each group were counted

to calculate the positive score of the NF-κB and CUGBP1.

1.5 The expression of NF-κB and CUGBP1 was detected quantitatively by Western Blot

The concentration of LPS, NNK and LPS combined NNK we used were based on the result of MTT assay. BMSCs were incubated with LPS12.5µg/ml, NNK10µg/ml, LPS12.5µg/ml combined NNK10 µg/ml for 24h, 48h, 96h respectively, then treated with RIPA (400µl/flask) containing PMSF on ice for cell lyses. The supernatants were collected after centrifugation (4°C10000r/min, 20min) and used as total protein extracts. After the concentration adjusted and denaturation in loading buffer (100°C water bath 5min). Total protein were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1% bovine serum albumin for 2h at room temperature, rinsed several times with TBST, then incubated with primary antibody (Rabbit anti Mouse NF-κB p65 polyclonal antibody, Rabbit anti Mouse CUGBP1 polyclonal antibody) overnight at 4°C, rinsed several

times with TBST, HRP-conjugated Goat anti Rabbit IgG was used as secondary antibody. Chemiluminescent signals were detected with ECL Plus western blotting detection reagents. The belts of all groups were analyzed by Image-pro plus software and obtained optical density value of each belt; the ratio of target bands and the band of GAPDH were calculated for statistical analysis.

1.6 Statistical Analyses

Data were represented as mean±standard deviation (mean±SD) and analyzed by SPSS 17.0. The difference was considered significant when $P < 0.05$.

2 Results

2.1 The morphological characteristics of BMSCs

Bone marrowstromal cells presented clone-like growth, showing a different size and irregular cell colony. Dense cells in the center of the colony and radiated-like growth at the peripheral area of the colony on the 7th day could be observed, overlap phenomenon was observed between colonies. The cells distributed uniformly after passage.

2.2 The effect of LPS, NNK and LPS combined NNK on the proliferation of mouse BMSCs

The proliferation activities of BMSCs incubated with LPS (12.5µg/ml, 25µg/ml and 50µg/ml), NNK (5µg/ml, 10µg/ml and 20µg/ml) and LPS combined NNK for 96h were detected by MTT assay and compared.

There was no significant difference between the group of LPS50 and control ($t_{\text{control, LPS50}}=3.578$, $P > 0.05$). However, both LPS12.5 and LPS25 could significantly promote the proliferation of BMSCs compared with control or LPS50 group ($t_{\text{control, LPS25}}=10.22$, $t_{\text{control, LPS12.5}}=12.33$, $t_{\text{LPS50, LPS25}}=6.641$, $t_{\text{LPS50, LPS12.5}}=8.752$, $P < 0.05$), but no significant differences could be seen between LPS12.5 and LPS25 ($t_{\text{LPS25, LPS12.5}}=2.111$, $P > 0.05$). NNK at the three concentrations could significantly promote the proliferation of mouse BMSCs compared with control ($t_{\text{control, NNK20}}=15.95$, $t_{\text{control, NNK10}}=17.23$, $t_{\text{control, NNK5}}=14.88$, $P < 0.05$), but no

significant differences could be seen among them ($t_{\text{NNK20, NNK10}}=1.273$, $t_{\text{NNK20, NNK5}}=1.074$, $t_{\text{NNK10, NNK5}}=2.234$, $P > 0.05$). There were no significant differences between the group of LPS50 combined NNK20 and control ($t_{\text{control, LPS50+NNK20}}=3.181$, $P > 0.05$), but other concentrations of LPS combined NNK could significantly promote the proliferation of mouse BMSCs compared with control and LPS50 combined NNK20 group ($t_{\text{control, LPS50+NNK10}}=8.963$, $t_{\text{control, LPS50+NNK5}}=8.983$, $t_{\text{control, LPS25+NNK20}}=9.863$, $t_{\text{control, LPS25+NNK10}}=10.54$, $t_{\text{control, LPS25+NNK5}}=8.222$, $t_{\text{control, LPS12.5+NNK20}}=11.16$, $t_{\text{control, LPS12.5+NNK10}}=7.602$, $t_{\text{control, LPS12.5+NNK5}}=9.463$, $t_{\text{LPS50+NNK20, LPS50+NNK10}}=5.782$, $t_{\text{LPS50+NNK20, LPS50+NNK5}}=5.802$, $t_{\text{LPS50+NNK20, LPS25+NNK20}}=6.682$, $t_{\text{LPS50+NNK20, LPS25+NNK10}}=7.362$, $t_{\text{LPS50+NNK20, LPS25+NNK5}}=5.042$, $t_{\text{LPS50+NNK20, LPS12.5+NNK20}}=7.982$, $t_{\text{LPS50+NNK20, LPS12.5+NNK10}}=4.421$, $t_{\text{LPS50+NNK20, LPS12.5+NNK5}}=6.282$, $P < 0.05$). But no significant differences could be seen when they were compared each other ($t_{\text{LPS50+NNK10, LPS50+NNK5}}=0.02001$, $t_{\text{LPS50+NNK10, LPS25+NNK10}}=1.580$, $t_{\text{LPS50+NNK10, LPS25+NNK20}}=0.9003$, $t_{\text{LPS50+NNK10, LPS25+NNK5}}=0.7402$, $t_{\text{LPS50+NNK10, LPS12.5+NNK20}}=2.20$, $t_{\text{LPS50+NNK10, LPS12.5+NNK10}}=1.360$, $t_{\text{LPS50+NNK10, LPS12.5+NNK5}}=0.50$, $t_{\text{LPS50+NNK5, LPS25+NNK20}}=0.8803$, $t_{\text{LPS50+NNK5, LPS25+NNK10}}=1.560$, $t_{\text{LPS25+NNK10, LPS12.5+NNK20}}=0.6202$, $t_{\text{LPS50+NNK5, LPS12.5+NNK20}}=2.181$, $t_{\text{LPS50+NNK5, LPS12.5+NNK10}}=1.380$, $t_{\text{LPS50+NNK5, LPS12.5+NNK5}}=0.4801$, $t_{\text{LPS25+NNK20, LPS25+NNK10}}=0.6802$, $t_{\text{LPS25+NNK20, LPS25+NNK5}}=1.640$, $t_{\text{LPS25+NNK20, LPS12.5+NNK20}}=1.300$, $t_{\text{LPS25+NNK20, LPS12.5+NNK10}}=2.261$, $t_{\text{LPS25+NNK20, LPS12.5+NNK5}}=0.4001$, $t_{\text{LPS25+NNK10, LPS25+NNK5}}=2.321$, $t_{\text{LPS50+NNK5, LPS25+NNK5}}=0.6202$, $t_{\text{LPS25+NNK10, LPS12.5+NNK10}}=2.941$, $t_{\text{LPS25+NNK10, LPS12.5+NNK5}}=1.080$, $t_{\text{LPS25+NNK5, LPS12.5+NNK20}}=2.941$, $t_{\text{LPS25+NNK5, LPS12.5+NNK10}}=0.6202$, $t_{\text{LPS25+NNK5, LPS12.5+NNK5}}=1.240$, $t_{\text{LPS12.5+NNK20, LPS12.5+NNK10}}=3.561$, $t_{\text{LPS12.5+NNK20, LPS12.5+NNK5}}=1.701$, $t_{\text{LPS12.5+NNK10, LPS12.5+NNK5}}=1.861$, $P > 0.05$).

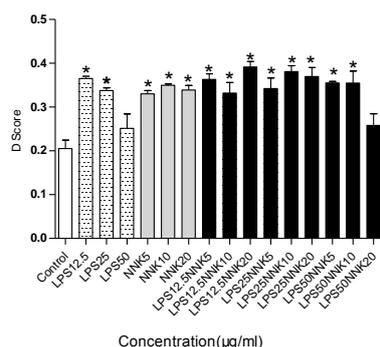


Fig.1 The effect of LPS, NNK and LPS combined NNK on the proliferation of BMSCs

2.3 The effect of LPS, NNK and LPS combined NNK on the morphological characteristics of NF-κB, CUGBP1 expression in BMSCs

2.3.1 The effect of LPS on the expression of NF-κB, CUGBP1 in mouse BMSCs

The expression of NF-κB and CUGBP1 in BMSCs incubated with LPS (12.5µg/ml) for 24h, 48h and 96h were detected and compared.

The positive expression of NF-κB in the control group was few and mainly in cytoplasm of BMSCs. After incubation with LPS for 24h, 48h, 96h respectively the positive expression of NF-κB were significantly increased in both cytoplasm and nucleus ($t_{\text{control, LPS24h}}=13.79$, $t_{\text{control, LPS48h}}=44.93$, $t_{\text{control, LPS96h}}=6.316$, $P < 0.05$). However, the positive expression of NF-κB in BMSCs incubated with LPS for 48h was mainly expressed in almost all nucleus, but mainly expressed in cytoplasm and relatively few in nucleus after incubated with LPS for 24h, 96h (Figure 2 B1~3). The positive score of LPS48h was higher than LPS24h and LPS96h, there were significant differences between group 48h and 24h, 96h ($t_{\text{LPS48h, LPS24h}}=31.14$, $t_{\text{LPS48h, LPS96h}}=38.61$, $P < 0.05$). The positive score of LPS24h was significant higher than LPS96h ($t_{\text{LPS24h, LPS96h}}=7.477$, $P < 0.05$) (Figure 4).

Almost negative expression or very few of CUGBP1 was expressed in BMSCs in the control group. After incubation with LPS the positive expression of

CUGBP1 could be observed in the cytoplasm and nucleus, mainly in nucleus in some BMSCs (Figure 3 F1~3). The positive expression of CUGBP1 in BMSCs incubated with LPS for 24h, 48h, 96h respectively and the number of BMSCs with CUGBP1 positive nucleus were significantly increased with the elongation of incubating time ($t_{\text{control, LPS24h}}=11.34$, $t_{\text{control, LPS48h}}=12.50$, $t_{\text{control, LPS96h}}=18.30$, $P < 0.05$). The positive expression of CUGBP1 and the number of BMSCs with CUGBP1 positive nucleus in 96h group was much more significantly increased than that in 24h and 48h group, and significant differences could be seen between them ($t_{\text{LPS24h, LPS96h}}=6.965$, $t_{\text{LPS48h, LPS96h}}=5.804$, $P < 0.05$), but no significant differences between 24h group and 48h group ($t_{\text{LPS48h, LPS24h}}=1.161$, $P > 0.05$) (Figure 4).

2.3.2 The effect of NNK on the expression of NF-κB, CUGBP1 in mouse BMSCs

The expression of NF-κB and CUGBP1 in BMSCs incubated with NNK (10µg/ml) for 24h, 48h and 96h were observed and compared.

After incubation with NNK for 24h, 48h, 96h respectively, the positive expression of NF-κB were significantly increased in both cytoplasm and nucleus. NF-κB in BMSCs incubated with NNK for 48h was mainly expressed in nucleus and mainly expressed in cytoplasm of BMSCs incubated with NNK for 24h and 96h (Figure 2 C1~3). The positive expression of NF-κB treated with NNK for 24h, 48h, 96h were significantly higher than that in control group ($t_{\text{control, NNK24h}}=21.77$, $t_{\text{control, NNK48h}}=28.36$, $t_{\text{control, NNK96h}}=6.598$, $P < 0.05$). The positive score of NNK48h was higher than control, NNK24h and NNK96h, there were significant differences between groups 48h and control, 24h, 96h ($t_{\text{NNK48h, NNK24h}}=6.598$, $t_{\text{NNK48h, NNK96h}}=15.17$, $P < 0.05$). The positive score of NNK96h was lower than NNK24h, NNK48h but higher than control, significant differences could be seen when compared with control, NNK24h and NNK48h ($t_{\text{NNK48h, NNK96h}}=11.65$, $t_{\text{NNK24h, NNK96h}}=21.77$, $P < 0.05$) (Figure 4).

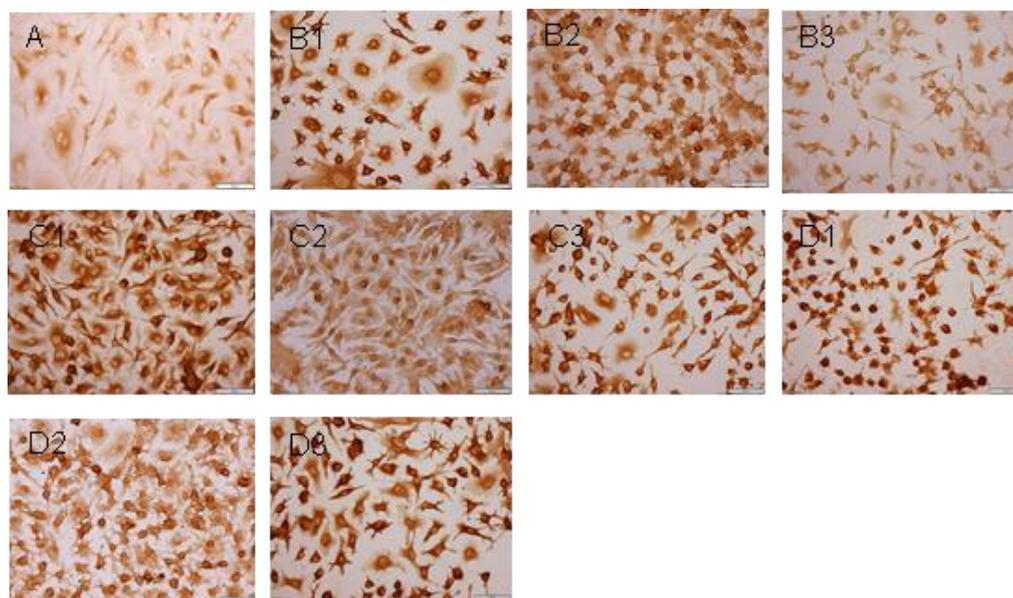
The expression of CUGBP1 was increased in BMSCs treated with NNK compared with that in control group. CUGBP1 were mainly expressed in cytoplasm of BMSCs incubated with NNK for 24h, but mainly expressed in nucleus after incubated for 48h and 96h (Figure3 G1~3). CUGBP1 expression with NNK for 24h, 48h, 96h were much higher than that in control group and there were significant differences ($t_{\text{control, NNK24h}}=8.899$, $t_{\text{control, NNK48h}}=14.39$, $t_{\text{control, NNK96h}}=14.74$, $P<0.05$). The positive score of CUGBP1 positive expression for NNK48h and NNK96h were higher than NNK24h, significant differences could be observed ($t_{\text{NNK24h, NNK96h}}=5.846$, $t_{\text{NNK48h, NNK24h}}=5.494$, $P<0.05$). But there were no significant differences between the NNK48h and NNK96h ($t_{\text{NNK48h, NNK96h}}=0.3522$, $P>0.05$) (Figure 4).

2.3.3 The effect of LPS combined NNK on the expression of NF- κ B, CUGBP1 in mouse BMSCs

The expression of NF- κ B and CUGBP1 in BMSCs incubated with LPS (12.5 μ g/ml) combined NNK (10 μ g/ml) for 24h, 48h and 96h were observed and compared.

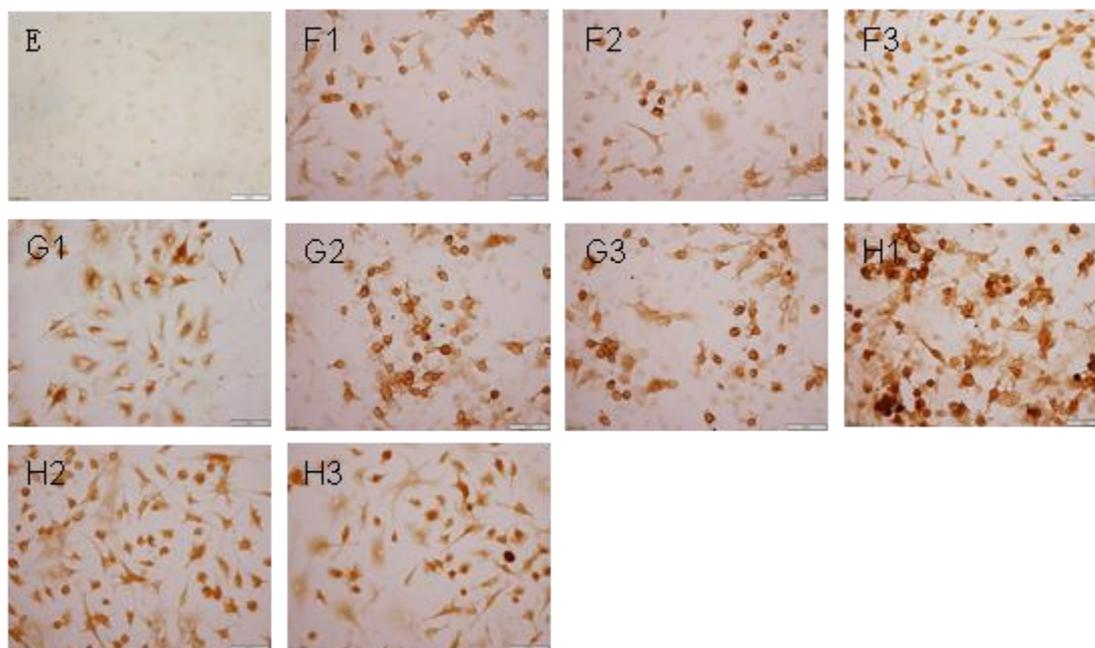
The positive expression of NF- κ B in cytoplasm and nucleus of BMSCs were significantly increased after incubated with LPS combined NNK for 24h, 48h, 96h respectively compared with control ($t_{\text{control, LPS+NNK24h}}=18.05$, $t_{\text{control, LPS+NNK48h}}=28.34$, $t_{\text{control, LPS+NNK96h}}=10.63$, $P<0.05$). In 24h group, NF- κ B were much higher expressed in nucleus than 48h and 96h group (Figure2 D1~3), significant differences were observed between them ($t_{\text{LPS+NNK48h, LPS+NNK24h}}=10.29$, $t_{\text{LPS+NNK48h, LPS+NNK96h}}=17.71$, $P<0.05$).

The expression of CUGBP1 in BMSCs treated with LPS combined NNK were mainly expressed in nucleus and increased compared with that in control group ($t_{\text{control, LPS+NNK24h}}=17.82$, $t_{\text{control, LPS+NNK48h}}=11.75$, $t_{\text{control, LPS+NNK96h}}=11.58$, $P<0.05$). The number of CUGBP1 positive nucleus in 24h group were much higher than that in 48h and 96h group ($t_{\text{LPS+NNK24h, LPS+NNK48h}}=6.074$, $t_{\text{LPS+NNK24h, LPS+NNK96h}}=6.242$, $P<0.05$), but no significant differences could be found between 48h and 96h ($t_{\text{LPS+NNK48h, LPS+NNK96h}}=0.1687$, $P>0.05$) (Figure 3 H1~3).



A: BMSCs were exposed to control; B1-B3: BMSCs were exposed to LPS (12.5 μ g/ml) for 24h, 48h, 96h; C1-C3: BMSCs were exposed to NNK (10 μ g/ml) for 24h, 48h, 96h; D1-D3: BMSCs were exposed to LPS (12.5 μ g/ml) combined NNK (10 μ g/ml) for 24h, 48h, 96h;

Fig.2 Expression of NF- κ B in BMSCs was detected by Immunocytochemistry



E: BMSCs were exposed to control; F1-F3: BMSCs were exposed to LPS (12.5µg/ml) for 24h, 48h, 96h; G1-G3: BMSCs were exposed to NNK (10µg/ml) for 24h, 48h, 96h; H1-H3: BMSCs were exposed to LPS (12.5µg/ml) combined NNK (10µg/ml) for 24h, 48h, 96h;

Fig.3 Expression of CUGBP1 in BMSCs was detected by Immunocytochemistry

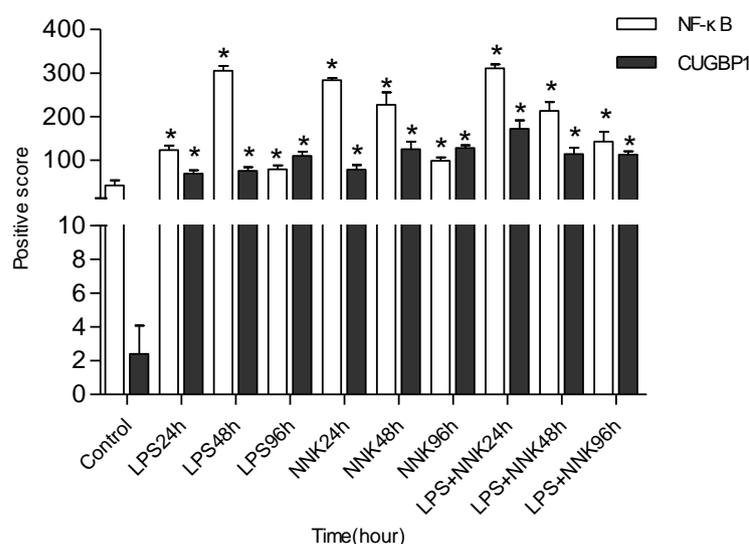


Fig.4 The positive score of the NF-κB and CUGBP1

2.4 The effect of LPS, NNK and LPS combined NNK on the expression level of NF-κB, CUGBP1 in BMSCs

The expression level of NF-κB, CUGBP1 in BMSCs incubated with LPS (12.5µg/ml), NNK (10µg/ml) and LPS (12.5µg/ml) combined NNK (10µg/ml) respectively

for 24h, 48h and 96h were quantitatively detected by Western Blot assay.

The results revealed that the expression level of NF-κB in different treatment groups were significantly increased when compared with control, ($t_{control, LPS24h}=3.585$, $t_{control,$

LPS48h=3.585, $t_{\text{control, NNK24h}}=15.91$, $t_{\text{control, NNK48h}}=9.404$, $t_{\text{control, NNK96h}}=4.099$, $t_{\text{control, LPS+NNK24h}}=11.57$, $t_{\text{control, LPS+NNK48h}}=5.546$, $t_{\text{control, LPS+NNK96h}}=4.822$, $P < 0.05$), except LPS96h group ($t_{\text{control, LPS96h}}=3.136$, $P > 0.05$). The expression level of NF- κ B in the group of LPS48h was higher than LPS24h and LPS96h. In the group of NNK24h, the expression level of NF- κ B was higher than NNK48h and NNK96h. The expression level of NF- κ B LPS combined NNK24h was higher than LPS combined NNK48h and LPS combined NNK96h. The expression level of NF- κ B in the group of NNK and LPS combined NNK were gradually lower with incubated time went on. The highest expression level of NF- κ B was shown in the group of NNK24h, and had significantly differences when compared with other groups ($t_{\text{NNK24h, LPS24h}}=12.06$, $t_{\text{NNK24h, LPS48h}}=10.37$, $t_{\text{NNK24h, LPS96h}}=12.78$, $t_{\text{NNK24h, NNK48h}}=6.510$, $t_{\text{NNK24h, NNK96h}}=11.81$, $t_{\text{NNK24h, LPS+NNK24h}}=4.340$, $t_{\text{NNK24h, LPS+NNK48h}}=10.37$, $t_{\text{NNK24h, LPS+NNK96h}}=11.09$, $P < 0.05$) (Figure 5, 6).

LPS48h=4.169, $t_{\text{control, LPS96h}}=7.926$, $t_{\text{control, NNK24h}}=6.061$, $t_{\text{control, NNK48h}}=8.859$, $t_{\text{control, NNK96h}}=6.061$, $t_{\text{control, LPS+NNK24h}}=12.59$, $t_{\text{control, LPS+NNK48h}}=6.072$, $t_{\text{control, LPS+NNK96h}}=6.994$, $P < 0.05$), except LPS24h group ($t_{\text{control, LPS24h}}=2.331$, $P > 0.05$). The expression level of CUGBP1 in BMSCs incubated with LPS was gradually higher with the elongated incubating time. In the group of NNK treatment, the highest expression level of CUGBP1 was observed after incubated for 48h. In the group of LPS combined NNK, the highest expression level of CUGBP1 was observed after incubated for 24h and the expressed level of CUGBP1 gradually lower as time went on. The highest expression level of CUGBP1 in all the group was shown in LPS combined NNK treatment for 24h, significant difference could be seen compared with other groups ($t_{\text{LPS+NNK24h, LPS24h}}=10.26$, $t_{\text{LPS+NNK24h, LPS48h}}=8.393$, $t_{\text{LPS+NNK24h, LPS96h}}=4.663$, $t_{\text{LPS+NNK24h, NNK24h}}=6.528$, $t_{\text{LPS+NNK24h, NNK96h}}=6.534$, $t_{\text{LPS+NNK24h, LPS+NNK48h}}=6.528$, $t_{\text{LPS+NNK24h, LPS+NNK96h}}=6.517$, $P < 0.05$) (Figure 5, 6).

The expression level of CUGBP1 in different treatment groups were significant higher than control ($t_{\text{control,}}$

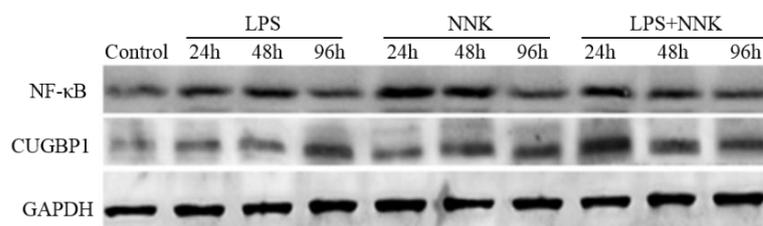


Fig.5 The expression of NF- κ B, CUGBP1 was detected quantitatively by Western Blot

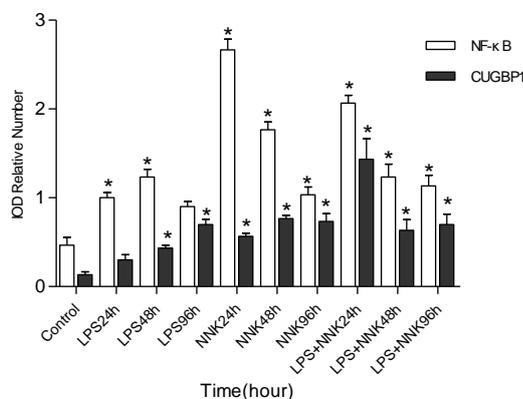


Fig.6 The IOD relative number of NF- κ B, CUGBP1

3 Discussions

Nuclear Factor κ B is a transcription factor that plays a key role in regulating the immune and inflammatory responses, oxidative stress responses, apoptosis, cell adhesion and could be activated by the stimulation of growth factors, cytokines and endotoxin, tumor promoter and oncogene, etc.^[11] In an inactive state, NF- κ B is sequestered in the cytoplasm as a heterotrimer consisting of p50, p65, and I κ B subunits and bound to its inhibitory molecule, I κ B α protein. Upon stimulation, I κ B α undergoes phosphorylation and ubiquitination-dependent degradation leading to p65 nuclear translocation and binding to a specific consensus sequence in the DNA^[12-13]. Activated NF- κ B binds to specific DNA sequences and regulates the expression of its target genes, leads to the expression of the downstream signal which may be caused the variation of cell proliferation^[14]. Recently studies showed that the migration and proliferation ability of breast cancer cells would be suppressed after NF- κ B signaling pathways were inhibited^[15], miR-451 over expression inhibits cell proliferation by inhibiting the NF- κ B signaling pathway through the direct suppression of IKK- β ^[16].

In this paper, we demonstrated that LPS at the concentration of 12.5 μ g/ml, 25 μ g/ml could significantly promote the proliferation of BMSCs, but LPS at the concentration of 50 μ g/ml could not promote the proliferation of BMSCs, this suggested that low concentration of LPS could promote the proliferation of BMSCs, but high concentration of LPS could not; NNK at all the three concentrations (5 μ g/ml, 10 μ g/ml and 20 μ g/ml) could significantly promote the proliferation of BMSCs and no significant different could be seen between them, this suggested the effect of NNK on the proliferation of BMSCs having nothing with NNK concentration, but closely related to the chemical structure; Our study further observe the effect of different concentration of LPS combined NNK on the proliferation of BMSCs, the results shown that all the combinations of LPS and NNK could significantly promote the proliferation of

BMSCs and no significant difference could be seen between different combinations, except LPS50 μ g/ml combined NNK20 μ g/ml. This suggested that cell proliferation could not be further promoted with the concentration of LPS and NNK increasing.

The expression of transcription factor NF- κ B (p65) was observed under different conditions for exploring the mechanism of the cell proliferation, our study revealed that the expression of NF- κ B could significantly promoted by LPS, NNK, LPS combined NNK; the translocation of NF- κ B complex into the nucleus after incubated with LPS or NNK for 48h, but the same situation was observed after 24h when incubate with LPS combined NNK. The translocation of NF- κ B complex into the nucleus plays a key role in the regulation of its target genes expression. The results suggested that the progress of physiology or pathology caused by stimulation of LPS combined NNK could accelerate the proliferation of BMSCs.

The CUGBP family proteins are major sequence-specific RNA binding proteins that control alternative splicing and mRNA translation and stability, which can regulate the proliferation and apoptosis of cells by affecting the stability of the mRNA. CUGBP1 as one of the regulators of CD9 expression and inversely correlated with CD9, the down-regulation of CD9 levels has been associated with the negative progression and metastasis outbreak of multiple cancer type, including non-small cell lung cancer, colon, oral squamous cell carcinoma, breast, acute myeloblastic leukemia, prostate, and B-acute lymphoblastic leukemia^[17-19]. Some papers suggest that over expression of CUGBP 1 in tumors reduces the expression of pro-apoptotic genes that are essential for cell death, thereby affecting the balance between proliferation and apoptosis^[20], and promote glioma cell proliferation by down-regulation of CDKN1B expression in glioma^[21], CUGBP1 mediated elevation of C/EBP β -HDAC1 complexes at the late stages of liver cancer eliminates p53-dependent inhibition of liver proliferation^[22]. Previous study revealed CUGBP1

was expressed in cytoplasm and nucleus of 95% A549 cells, EGCG can inhibit the proliferation of A549 cells by down-regulation of CUGBP1 expression. Inflammatory stimuli factor LPS can promote the proliferation of A549 cells and expression of CUGBP1 in its nucleus. This suggested the expression increase of CUGBP1 may be related to the proliferation of A549 cells and inflammatory response; LPS could significantly enhance the carcinogenesis of lung stimulated by NNK *in vivo*, but the mechanism is not clear [23]. In this study, the effect of LPS, NNK and LPS combined NNK on the expression of CUGBP1 in BMSCs was detected. Our result revealed that the expression of CUGBP1 was very few or negative in BMSCs in control group. The CUGBP1 expression was significantly increased in BMSCs treated with LPS and NNK respectively, the highest level of CUGBP1 protein expression was shown at 96h for LPS incubation and 48h for NNK incubation. Furthermore, the highest level of CUGBP1 protein expression in BMSCs treated with LPS combined NNK was detected at 24h. The results suggested that CUGBP1 could not be expressed in BMSCs cultured *in vitro*. LPS, NNK could not only facilitate BMSCs proliferation but also activate BMSCs to express CUGBP1 respectively and the role of NNK is stronger than LPS. We were surprised to find that when LPS and NNK were used in combination, the CUGBP1 expression in BMSCs was significantly ahead of time, which maybe could explain the reason for the role of LPS in promoting NNK induced lung cancer *in vivo*, and it is also possible to further explain the causes of lung cancer in smokers and why the inflammatory reaction is closely related to the occurrence and development of tumor. That maybe mediated by the mechanism of affecting the stability of mRNA. However, Whether BMSCs transform the tumor cells under the condition of longterm LPS and NNK stimulation need to explore further.

Increasing evidence supports Chronic inflammation has been found to mediate a wide variety of diseases, including cancer, Chronic inflammation has been linked to various steps involved in tumorigenesis,

including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. Chronic inflammation is accompanied by increased production of tissue reactive oxygen and nitrogen intermediates. The pre-neoplastic activity of reactive oxygen species is mainly due to their ability to cause DNA damage. Proteins and lipids are also significant targets for oxidative attack, and modification of these molecules in a developing tumor microenvironment can increase the risk of mutagenesis [24~26].

The results showed that peak expression and nuclear transfer of NF- κ B in BMSCs stimulated by LPS and NNK respectively were in a time; but the peak expression of CUGBP1 was lag than that of NF- κ B and its nuclear transfer. When BMSCs treated with LPS combined NNK, the peak of NF- κ B expression and its nuclear transfer was consistent with the peak of CUGBP1 expression. The relationships between NF- κ B and CUGBP1 in the process of BMSCs proliferation need a further study.

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