ORIGINAL ARTICLE

Effects of MG-132 in Mice with Inflammatory Acute Viral Myocarditis

XM Zhang[1, 2], YC Li[1], S Ye[1], YH Chen[1], XJ Yang[2], P Chen[1]

ABSTRACT

Objective: *To determine the effects of ubiquitin proteasome inhibitor MG-132 in mice with inflammatory acute viral myocarditis induced by coxsackie virus B3 (CVB3) infection.*

Methods: *BALB/C mice were intraperitoneally inoculated with CVB3 to induce myocarditis. Twenty-four hours after infection, MG-132 was administered at a dose of 0.5 mg/kg or 1 mg/kg for seven days by intraperitoneal injection. Normal controls were treated with the same volume of DMSO. The changes in myocardial ultrastructure, the protein levels of IL-6 and TNF-α, and the number of polymorphonuclear leucocytes (PMN) and survival rate of each group were documented.*

Results: *Compared with the normal group, the protein levels of IL-6 and TNF-α, and the number of PMN were significantly increased in the CVB3 group (*$p < 0.05$*). The cardiocytes were diffusely swollen. The myofilaments were lysed, and the mitochondria swollen and vacuolizated. The above-mentioned inflammatory factors of the MG-132 group were significantly decreased when compared with the CVB3 group (*$p < 0.05$*). The degree of the damage of the cardiocytes in the MG-132 group was less, and the mortality due to deadly arrhythmia was lower than that in the CVB3 group.*

Conclusion: *The results showed that MG-132 protected the mice from CVB3-induced acute viral myocarditis by suppressing the expression of the inflammatory factors. Our research provides further evidence that the ubiquitin-proteasome system is a potential target therapy for viral myocarditis.*

Keywords: IL-6, inflammatory factors, MG-132, polymorphonuclear leucocytes, TNF-α, viral myocarditis

Efectos del MG-132 en ratones con miocarditis viral inflamatoria aguda

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RESUMEN

Objetivo: *Determinar los efectos del MG132, inhibidor del sistema ubiquitina-proteosoma, en ratones con miocarditis viral inflamatoria aguda inducida por infección del virus Coxsackie B3 (CVB3).*

Métodos: *Ratones BALB/C fueron inoculados con CVB3 por vía intraperitoneal, con el fin de inducirles miocarditis. Veinticuatro horas después de la infección, se les administró MG-132 en dosis de 0.5 mg/kg ó 1 mg/kg durante siete días mediante inyección intraperitoneal. Los controles normales fueron tratados con el mismo volumen de dimetil sulfóxido (DMSO). Los*

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cambios en la ultraestructura miocárdica, los niveles de proteína de IL-6 y TNF-α, el número de PMN, y la tasa de supervivencia de cada grupo fueron documentados.
Resultados: *En comparación con el grupo normal, los niveles de proteína de IL-6 y TNF-α, y el número de leucocitos polimorfonucleares (PMN) aumentaron significativamente en el grupo de CVB3 (*$p < 0.05$*). Los cardiocitos estaban difusos e hinchados, los miofilamentos estaban rotos, y las mitocondrias hinchadas y vacuolizadas. Los mencionados factores inflamatorios del grupo MG-132 disminuyeron significativamente en comparación con el grupo de CVB3 (*$p < 0.05$*). El grado de daño de los cardiocitos en el grupo de MG-132 fue menor, y la mortalidad debido a la arritmia mortal fue menor que en el grupo de CVB3.*
Conclusión: *Estos resultados mostraron que el MG-132 protegía a los ratones con miocarditis viral aguda inducida por CVB3, en la medida en que suprimía la expresión de los factores inflamatorios. Nuestra investigación proporciona evidencia adicional en cuanto a que el sistema ubiquitina-proteosoma constituye una potencial terapia de elección para la miocarditis viral.*

Palabras claves: IL-6, factores inflamatorios, MG-132, leucocitos polimorfonucleares, TNF-α, miocarditis viral

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INTRODUCTION

The ubiquitin-proteasome system (UPS) is the main route of intracellular ATP-dependent protein selective degradation, and it is an essential control system in intra-eukaryocytes, which plays a role in maintaining cellular homeostasis for it is involved in cellular apoptosis, inflammatory reactions, cellular cycle and signal transduction (1−3).

Coxsackie virus B3 (CVB3) can induce viral myocarditis, and in the acute phase, it induces myocardial inflammation and necrosis, complicated with heart failure and arrhythmia, which leads to sudden death or dilated cardiomyopathy. However, there is no available treatment for viral myocarditis (4). Recent studies have revealed that UPS plays a key role in viral infected conditions for it takes part in the inflammatory reaction (5−7). Moreover, it plays a role in the inflammatory reaction process of a few diseases (8−10). Therefore, this study was done to investigate whether ubiquitin proteasome inhibitor, MG-132, is effective in the inflammation induced by CVB myocarditis, and the possible mechanism.

SUBJECTS AND METHODS

Animals and protocols

Four-week-old male inbred mice, specific-pathogen free and 20 ± 5 g by weight, were purchased from Zhejiang Province Experimental Animal Centre. Eighty mice were randomly divided into four groups (n = 20): sham group, myocarditis group (CVB group), myocarditis + MG-132 low dosage group (CVB + T1 group), and myocarditis + MG-132 high dosage group (CVB + T2 group).

Reagents

Coxsackie virus B3 viral solution (10^7 TCID 50/0.1 mL titer, 1 mg/kg) was provided by Calbiochem Corporation (USA) and was diluted with 0.2% DMSO.

Drug administration and specimen collection

Mice with myocarditis were injected with 0.1 mL CVB3 (100 TCID 50/0.1 mL) intraperitoneally, and the mice of the sham group were injected with 0.1 mL phosphate buffer solution. The next day, the mice of CVB + T1 and CVB + T2 groups were injected with 0.5 mg/kg and 1.0 mg/kg MG-132 intraperitoneally, and the mice of the CVB group were given DMSO intraperitoneally for seven days, once per day. One day after the seventh administration, blood was collected through the venous plexus of the eyes. The heart samples were collected for pathological tests, electron microscope examination and real-time polymerase chain reactions before cardiac arrest.

Histopathological test

Ten per cent formaldehyde-fixed hearts were dehydrated, embedded in paraffin and then cut into slices. The pathological changes were determined by the average value of the microscopic examinations of two researchers. The criterion was: if the area with inflammatory infiltration or myocardial necrosis took up < 25% of the slice, it was

scored one; if it took up 25–50%, it was scored two; if it took up 51–75%, it was scored three; and if it took up > 75%, it was scored four. Five fields of view (× 400) were picked randomly for each slice, and the numbers of polymorphonuclear leucocytes (PMN) were counted, then the average value of the five numbers was used.

Transmission electron microscope

The hearts were fixed firstly in 2.5% glutaraldehyde and then in 1% osmic acid. The samples were dehydrated gradually in acetone and embedded in epoxy Epon 812. After the samples were cut into slices using ultra microtome, the slices were dyed in 1% uranium acetate and lead citrate and observed by JEM-1200EX transmission electron microscope.

Determination of protein levels of TNF-α and IL-6 by enzyme-linked immunosorbent assay

Serum was collected for TNF-α and IL-6 level determination by the protocols of corresponding enzyme linked immunosorbent assay (ELISA) kits bought from Shanghai Yikesai Biology Technology Company.

Determination of mRNA levels of TNF-α and IL-6 using fluorescent quantitative polymerase chain reaction

The whole ribonucleic acid (RNA) was extracted with Trizol. The procedures of reverse transcription and fluorescent quantization were followed according to the respective kit's protocol. F = 2^–(average value of target gene Ct of the group tested – average value of control gene Ct of the group tested) – (average value of target gene Ct of all groups – average value of control gene Ct of all groups).

Statistical analysis

The experimental results were expressed as mean ± standard deviation ($\overline{x} \pm s$). The data were analysed using inter-group variance analysis, and t-test using SPSS 17.0, and $p < 0.05$ was considered to be statistically significant.

RESULTS

Survival rate analysis

The survival rate of the sham group was 100% on the eighth day. The mice injected with CVB3 displayed less appetite and less movement, and their peak time to death was the fifth to the seventh day. MG-132 at the low dosage increased the survival rate of the CVB group (60% *vs* 45%). MG-132 at the high dosage increased the survival rate of the CVB group significantly (75% *vs* 45%), and also delayed the peak time to death.

Pathological changes of myocardial tissues under a microscope

As shown in Fig. 1, MG-132 lessened the pathological changes induced by myocarditis, and the high dosage decreased the inflammatory infiltration and myocardial necrosis significantly. The number of PMN of the sham group was 0.86 ± 0.32 / field of view, and in the CVB group, the number significantly increased to 33.7 ± 4.3 / field of view. The number of PMN of the CVB + T1 group was 13.5 ± 2.3 / field of view, and that of the CVB + T2 group was 9.7 ± 1.2 / field of view. In the CVB + T1 and CVB + T2 groups, the number of PMN in myocarditis decreased significantly ($p < 0.01$).

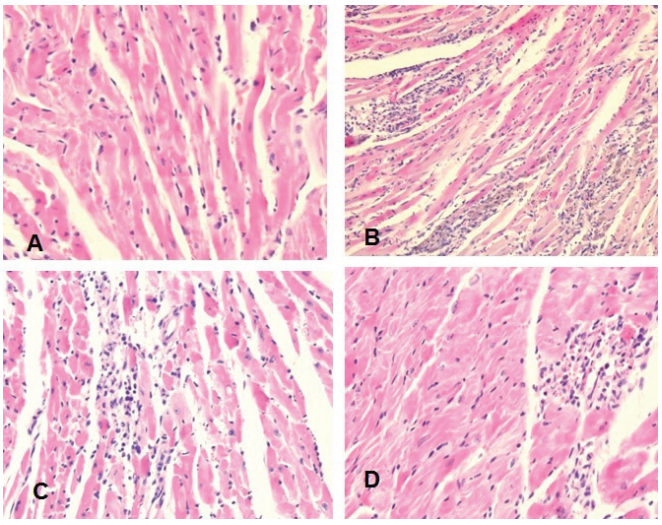

Fig. 1: Haematoxylin-eosin staining. A: sham group; B: CVB group; C: CVB + T1 group; D: CVB + T2 group.

Ultrastructure changes of myocardial tissues

As shown in Fig. 2, compared with the sham group, other groups demonstrated different ultrastructure changes in the myocardial tissues. Both CVB + T1 and CVB + T2 groups alleviated the destruction induced by myocarditis, and the CVB + T2 group lessened the destructive changes significantly.

mRNA levels of inflammatory factors IL-6 and TNF-α

Compared with the sham group, the mRNA levels of IL-6 and TNF-α were significantly higher ($p < 0.01$). The CVB + T1 group had much lower mRNA levels of IL-6 and TNF-α than the myocarditis group. The CVB

+ T2 group demonstrated significantly lower levels than the myocarditis group ($p < 0.05$) (Table 1 and Fig. 3).

Protein levels IL-6 and TNF-α measured by enzyme-related immunosorbent assay

Compared with the sham group, the protein levels of IL-6 and TNF-α were significantly higher ($p < 0.01$). Both CVB + T1 and CVB + T2 groups had decreased IL-6 and TNF-α expression levels of myocarditis, and the CVB + T2 group had significantly lowered IL-6 and TNF-α expression levels of myocarditis ($p < 0.01$) (Table 2).

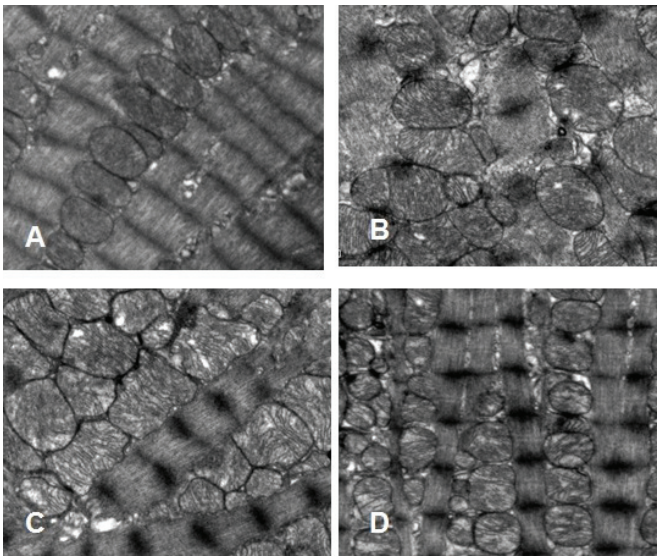

Fig. 2: Transmission electron microscope (×10 000). A (sham group): cardiac muscle fibrils were neatly arranged on the longitudinal section, and Z line divided myofibril into muscle sarcomere clearly; there were adequate mitochondria in myofibril, and they were round and arranged neatly. Intact ridges can be detected. B (CVB group): there was obvious destruction on ultrastructure. Broken myofibril and sarcomere were here and there, and mitochondria were arranged irregularly. C (CVB + T1 group): Z lines were rather vague, and parts of sarcomeres were destroyed. Mitochondria demonstrated hyperplasia and were swollen. D (CVB + T2 group): compared with the CVB group, the ultrastructures were alleviated. Z lines were rather vague, and sarcomere has a clear structure. Mitochondria demonstrated less hyperplasia and were less swollen.

DISCUSSION

There have been no available treatments for viral myocarditis, and some immunotherapeutic methods have little effect on the patients in random clinical trials. Therefore, any new breakthrough treatment for myocarditis is of importance worldwide.

The UPS is not only the regulation system of protein quality in intra-eukarycyte, but it is also involved in cellular apoptosis, inflammatory reaction, cellular cycle, intracellular transduction and antigen presentation. Recent studies have demonstrated that UPS plays an essential role in many viral infected diseases (5–7, 11). Viruses may make use of UPS to replicate more viruses and induce oxidative stress injury in the host cells (12, 13). Teale *et al* found that UPS was essential in the virus RNA transcription process of vaccinia virus (14). Raaben *et al* revealed that UPS was involved in

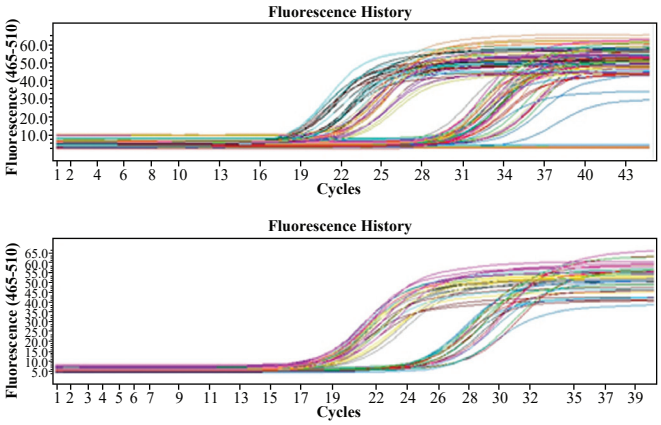

Fig. 3: Curves of polymerase chain reactions.

Table 1: IL-6, TNF-α mRNA levels in the myocardial tissues

Group	Sham	CVB	CVB + T1	CVB + T2
IL-6 mRNA	0.21 ± 0.02	1.85 ± 0.12*	1.59 ± 0.09*	1.06 ± 0.06*Δ
TNF-α mRNA	0.18 ± 0.03	1.38 ± 0.08*	1.21 ± 0.07*	0.76 ± 0.04*Δ

*Compared with sham group, $p < 0.01$; Δcompared with CVB group, $p < 0.05$; n = 20

Table 2: IL-6, TNF-α protein levels in the serum

Group	Sham	CVB	CVB + T1	CVB + T2
IL-6 level (pg/ml)	382 ± 66	4978 ± 916*	3486 ± 642*Δ	2132 ± 338*ΔΔ
TNF-α level (pg/ml)	467 ± 74	3263 ± 633*	2574 ± 514*Δ	1805 ± 267*ΔΔ

*Compared with sham group, $p < 0.01$; Δcompared with CVB group, $p < 0.05$; ΔΔcompared with CVB group, $p < 0.01$; n = 20

hepatitis B corona virus inflammatory injury (15). Lin *et al* found that TNF-α ubiquitin ligase was over-activated in the livers of rats with liver cirrhosis, and TNF-α ubiquitin ligase took part in the inflammatory reaction and protein degraded process (6).

Other studies revealed that UPS played a key role in inflammatory reactions for it was a part of the NF-κB pathway and inhibition of UPS reduced inflammatory factor levels, such as MMP13 and IL-6 (10). Qin *et al* found that UPS was involved in carotid atherosclerosis, and ubiquitin proteasome inhibitor PYR-41 lessened endarterial stenosis by alleviating TNF-α-induced inflammation and decreasing the expression level of apoptosis factor p53 (8). Lee *et al* also revealed that ubiquitin proteasome inhibitor MG-132 and B ortezomib inhibited inflammatory reaction, cardiac hypertrophy and cardiac failure through serine/threonine-specific protein kinase AKT and extracellular signal-related kinase (ERK) pathway (9).

The role of UPS in viral myocarditis has seldom been explored. Luo *et al* reported that UPS exacerbated the cardiac function of viral myocarditis, but its mechanism was not clear (5). In this study, mice were injected with CVB3 intraperitoneally to induce acute myocarditis; then, MG-132 was administered for seven days. The results demonstrated that MG-132 decreased the pathological changes in the hearts with myocarditis (16). Our research further revealed that MG-132 at different doses (0.5 mg/kg and 1.0 mg/kg) alleviated the pathological changes of cardiac tissues, inhibited inflammatory factor levels and PMN infiltration; and that a MG-132 high dosage (1.0 mg/kg) significantly lessened the pathological outcomes of myocarditis.

The UPS is very important in organisms, and its essential role is found in in-depth studies (17). This study used a CVB3 viral myocarditis model, and the results revealed that ubiquitin proteasome inhibitor MG-132 decreased the inflammatory factor levels and alleviated the myocardial injury of myocarditis. Therefore, ubiquitin proteasome inhibitor is likely to be a potential treatment for acute viral myocarditis.

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