Repairing effects of interleukin 11 (IL-11) towards high dose methotrexate-induced rat small intestinal mucositis and its impacts on T-lymphoblastic leukemia cell line

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A B S T R A C T

Objective(s): To investigate the efficacy of interleukin 11 (IL-11) towards the high dose methotrexate (HDMTX)-concurrent rat small intestinal mucositis and its impacts on the proliferation of the human T-lymphoblastic leukemia (CEM) cell line.
Materials and Methods: 95 Wistar rats were randomly divided into five groups, the normal control group (A), the methotrexate (MTX) control group (B), the IL-11-pre-treated high-dose group (C), the post-IL-11-treatment high-dose group (D) and the post-IL-11-treatment low-dose group (E). After the intraperitoneal injection of MTX in the groups B-E, the rats were sacrificed at 1, 3, 5 and 7 days. The mortality, morphological and ultrastructural changes of small intestine of each group were observed. The cells were then cultured in vitro, and the MTT method was used to investigate the effects of different concentration of IL-11 on CEM proliferation and also on HDMTX-induced mucositis.
Results: IL-11 could reduce the intestinal histopathological score, increase the height of small intestinal villi, promote the proliferation of intestinal lacunar cells and reduce the mortality rate of rats. The IL-11 pre-treatment group exhibited the best efficacies, demonstrating significant difference with the control group (P<0.01). In addition, the proliferation of CEM was not promoted, indicating that IL-11 could not inhibit HDMTX.
Conclusion: IL-11 could reduce the severity of HDMTX-induced intestinal mucositis, and improve the survival rate of experimental rats, and could be safely used as the adjuvant treatment of HDMTX in childhood leukemia.

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Introduction

The high dose methotrexate (HDMTX) chemotherapy had been widely used in the childhood. The HDMTX chemotherapy could not only be effective in preventing the occurrence of extramedullary leukemia, but also improve the disease-free survival of children with acute lymphocytic leukemia. Increasing the amount of methotrexate (MTX) can significantly increase the drug concentrations in the blood and cerebrospinal fluid, thus enhancing the efficacy, while the side effects were also increasingly prominent. As it is well known that MTX had higher affinities towards the tissues such as the bone marrow, skin and gastrointestinal mucosa due to their higher proliferation rate, shorter proliferation cycle (24-48 hr) and faster regeneration speed, hence, myelosuppression and gastrointestinal mucositis can be considered as the

main side effects of MTX. It has been reported that the incidence of mucositis could still be as high as 20-25% despite adequate preparation (1), and even up to 39% after HDMTX chemotherapy (2). The severe mucositis would not only affect the patients' eating, and prolong the hospitalization, but can also cause the bloody diarrhea, sepsis, septic shock, and even death, and as a consequence, can seriously influence the next course of chemotherapy. Currently, there is no effective strategy for the management of HDMTX chemotherapy side effects. How to maximally reduce the post-HDMTX incidence and severity of mucositis, and raise leukemia children's tolerance to HDMTX, had become the most important issue that needed to be solved.

In recent years, researchers had established a variety of MTX-induced mucositis model to reveal the pathological process of MTX-induced mucositis,

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thus providing the theoretical basis for the clinical treatment. Some studies have shown that a number of growth factors and certain amino acids may have mucosal protective effects through different mechanisms. For example, the hepatocyte growth factor, intestinal TFF3 factor and insulin-like growth factor (IGF-I) etc. can play their roles through the autocrine or paracrine mechanism; the keratinocyte growth factor (KGF) can enhance the DNA repair, thus affecting the proliferation and differentiation of intestinal epithelial stem cells; and the intestinal transforming growth factor can repress the cell cycle of intestinal lacunar stem cells, thus preventing the differentiation of intestinal epithelial cells in the G1 phase, so that the lacunar stem cells will not be damaged by the cell cycle-specific chemotherapeutic drugs (3-10).

The glutamine (GLN), not only provided the nutritional supports for the epithelium and mucosa, but also could increase the concentration of intracellular MTXPG and enhance anti-metabolic effects of MTX (11, 12). Findings show that the novel cytoprotective agent amifostine can prevent and treat the radiotherapy and chemotherapy-induced oral and intestinal mucositis (13, 14). However, the mechanisms of HDMTX-induced mucositis had not been elucidated. Also, it has been found that MTX can directly inhibit the DNA synthesis of epithelial cells or accelerate the apoptosis of intestinal crypt cells (15, 16), so that the risk of gut-associated sepsis may be increased (17). In addition, the age and inadequately-prepared chemotherapy might also increase the toxicity of MTX.

The researches about whether the hematopoietic growth factor interleukin 11 (IL-11) had the mucosal protective effects were rare. A few single-center experimental researches have been conducted on the mucositis model or cancer patients who also suffered from the mucositis, and found that IL-11 could reduce the severity and duration of mucositis, thus improving the quality of life in these patients (18-20). However, IL-11 was basically one hematopoietic growth factor derived from the bone marrow stromal cells, which played a major role in the regulation of hematopoiesis. Since, no study has been performed on whether it can promote the proliferation of tumor cells during stimulating the hemocytogenesis, and affect the function of anticancer drugs, in this study; a research was conducted to study this issue.

Materials and Methods

Animals and induction of experimental intestina mucositis

In this study, 95 Wistar rats, weighed 120-150 g were randomly divided into five groups: Group A (n=15): the normal control group; Group B (n=20): the MTX control group; Group C (n=20): the high-dose IL-11 (475 µg/kg^{-1}/d^{-1}, Qilu Pharmaceutical Co, Ltd, Jinan, China) treatment before using MTX (Jiangsu Hengrui

Medicine Co, Ltd, Lianyungang, China); Group D (n=20): the high-dose IL-11 (475 µg/kg^{-1}/d^{-1}) treatment after using MTX; Group E (n=20): the low-dose IL-11 (150 µg/kg^{-1}/d^{-1}) treatment after using MTX. A total of 95 5-week-old Wistar rats (male or female), clean grade, weighed 120-150 g were housed at room temperature (20-22 ℃) in 12/12 dark light cycles and 50-55% humidity.

The dose range of HD-MTX was based on a previous study (21), and the recommended dose was 3 g / m^2 (100 mg / kg) in terms of body weight. The dose of IL-11 referred to the package insert.

The group A was injected normal saline, with 1 ml/per rat from the 1st to 5th day, the group B-E were intraperitoneally injected 1 ml MTX (100 mg/kg) to establish the mucositis model. The Group B was intraperitoneally injected 1 ml MTX on the d$_0$ day; meanwhile, 1 ml saline was subcutaneously injected simultaneously with MTX, as well as 2 days before and after the injection, respectively. The Group C was subcutaneously injected high-dose IL-11 2 days before the MTX injection, twice/day × 2 days. The Group D was subcutaneously injected high-dose IL-11 two days after the MTX injection, twice/day × 2 days. The Group E was subcutaneously injected low-dose IL-11 two days after the MTX injection, twice/day × 2 days.

Two rats of the Group B were sacrificed on the 1st, 3rd, 5th and 7th day after the MTX injection, respectively, and 2 rats of the C, D and E group were sacrificed on the 3rd and 5th day after the MTX injection; the time points of the group A were the same as the group B. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Liaocheng People's Hospital.

Hematoxylin and eosin staining

The proximal segment of jejunal tissues were taken for pathological analysis and hematoxylin and eosin (HE) staining. each group was randomly selected two slices, and each slice was randomly selected three high-power fields, the calculation of histopathological score was in accordance with the Howarth *et al* method (4) for the semiquantitative analysis. No lesion was recorded as 0 point, the mild lesion was recorded as 1 point, the moderate lesion was recorded as 2 points, and the severe lesion was recorded as 3 points.

Determination of intestinal villus height, crypt depth

Determination of intestinal villus height, crypt depth and calculation of villus height/crypt depth ratio was performed. For this purpose, 3 pathological sections of 2 rats of each group were counted using 200 X light microscope, and 10 villus heights and 10 crypt depths were detected in each slice.

Immunohistochemical staining

The jejunal tissue paraffin sections were dewaxed and hydrated, followed by washing with phosphate buffered saline (PBS) for two times, 5 min for each time. After enzyme closure with 3% hydrogen peroxide at room temperature for 5-10 min, the antigen retrieval was performed. After washing with PBS for 5 min, the normal goat serum (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) was added for blocking at room temperature for 20 min. The excess liquid was removed, and then the primary antibody (Fuzhou Maixin Biotechnology Development Co, Ltd, Fuzhou, China) was added, followed by incubation at room temperature for 1 hr. After washing with PBS for 3 times, 2 min for each time, the biotinylated secondary antibody (Fuzhou Maixin Biotechnology Development Co, Ltd., Fuzhou, China) was added, followed by incubation at 20-37 °C for 20 min. After washing with PBS for 3 times, 2 min for each time, SABC was added, followed by incubation at 20-37 °C for 20 min. After coloration, counterstain and mounting, the sections were observed in Q550CW image acquisition and analysis system (Leica Science Lab, Berlin, Germany). The absolute values of positively-stained cells towards the total small-intestinal glandular cells, as well as the ratio of the entire positive nuclei were counted. The crypt and nuclei of 2 rats were counted with 400-600 nuclei and 10 similar crypts per rat.

Electron microscope

The proximal segment of jejunal tissues were sampled under the low temperature conditions to prepare the ultra-thin slice, and then the Lead-Uranium dual-staining electron microscope was used to observe the ultrastructural changes.

Mortality rates

The mortality rates of the experimental animals were investigated at the 1st, 3rd, 5th and 7th day after the HDMTX injection.

Proliferation of the human T-lymphoblastic leukemia (CEM) cells

The MTT (Sigma-Aldrich Corp., MO, USA) assay was performed to test the impacts of different concentrations of IL-11 on proliferation of CEM cells (Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin, China) in different time periods. CEM cells in the exponential growth phase (with the cell concentration at 3.0×10^5/ml) were collected. The final concentrations of IL-11 were 9.375, 18.75, 37.5, 75, 150, 300, 600, and 1200×10^{-3} μg/ml, respectively. One hundred microliter of various IL-11 concentrations together with 100 μl cell suspensions were added into the 96-well flat-bottom culture plates. Three repeating wells were set for each sample group (the experimental group, the cell control group and the control group) , and

the samples were cultured at 37 °C, 5% CO_2 and saturated humidity for 24, 48 and 72 hr. Ten microliter MTT reagent (5 mg/ml) was added into each well 4 hr before terminating the culture. Afterwards, the samples were centrifuged at 2000 r/min for 10 min. The supernatant was then discarded, and 150 μl DMSO (Beijing Asia-Pacific Chemical Technology Co, Ltd, Beijing, China) was added to each well, followed by the thorough shaking to completely dissolve the formazan particles. The absorbance was then measured with ELISA assay (BS-1101, Nanjing Tiede Experimental Equipment Co, Ltd, Nanjing, China) at 570 nm as the detection wavelength, and 630 nm as the reference wavelength, the $A_{570-630}$ was recorded as the final absorbance value.

Impacts of IL-11 on the anti-tumor effects of MTX

The CEM cells in the exponential growth phase (with the cell concentration as 3.0×10^5/ml) were taken. In the A group (MTX control group), the final concentrations of MTX were 6, 12.5, 25, 50, 100, and 200×10^{-3} μg/ml; but, in the B group (IL-11 + MTX groups), the final concentrations of IL-11 were 75, 150, 300, and 600×10^{-3} μg/ml, while the final concentrations of MTX were the same as the group A. The IL-11 control group and the control group were also set simultaneously. Then, IL-11 and MTX would act together on the CEM cells for 24 and 48 hr, respectively. The detection method was with ELISA assay. Subsequently, the inhibition rates of the two groups were compared. The inhibition rate (%)= (absorbance of the cell control group – absorbance of the experimental group)/absorbance of the cell control group × 100%.

Statistical methods

The SPSS 12.0 window statistical software was used to analyze the data, and the χ^2 test was used for the comparison of rates. In addition, t test was used to compare the mean values of two samples. Bilateral $P<0.05$ was considered as the statistically significant differences.

Results

Incidence and mortality

The A group exhibited normal eating activities, without diarrhea and death; while, the rats of B~E group exhibited different degrees of hair loosening, back arching, diarrhea, activity and eating reduction two days after the MTX injection. The diarrhea was more severe on the 3rd day, while the eating was increased from the 5th day, and the diarrhea gradually stopped on the 7th day. The anatomical specimen revealed that MTX injection group exhibited severe retention of gastrointestinal liquid, flatulence, intestinal vascular congestion and mesenteric adhesions on the 3rd day, especially in the B group. The mortality rate of the B group was higher than other groups (up to 50%), while that of

the C group was the lowest with only 15%, indicating that there was a significant difference between the 2 groups (*P*<0.01). The mortality rate of D group was also 20%, which exhibited significant difference with the B group (*P*<0.05). The comparison among the C, D and E groups exhibited no significant difference (*P*>0.05). Although the comparison between the E and B groups exhibited no statistically significant difference (χ^2=1.08, *P*>0.05), the mortality rate was still lower than the MTX control group.

Morphological changes

The A group exhibited no significant pathological changes in the intestinal tissues. The small intestine villi were slender, neatly arranged, and the brush-like border was clear. Also, the epithelial cells were intact, and the goblet cells were plenty. The B~E group exhibited different degrees of pathological changes in the intestinal tissues, among which the situation of the B group was more severe on the 3rd day. The intestinal villi were significantly shorter, flatter, atrophic, and even completely ablated. The lacunar morphology of small intestine was lost, and the goblet cells disappeared; moreover, the villous stroma exhibited congestion, with the infiltration of a large number of inflammatory cells, lacunar abscesses. Also, the cell proliferation was extremely reduced. The pathological changes were significantly reduced on the 5th day, the villi were increased, and the number of goblet cells was increased. The pathological changes of the C, D and E group, especially the C group (with the lightest pathological changes and the lowest pathological score) on the 3rd day were significantly lighter than the B group, exhibiting significant differences when compared with the B, C and D group (*P*<0.05). The villus height and the lacunar depth were greater than those of the B, D and E group at the same time point. The results of each group were shown in Table 1, 2 and 3. Also, the Figures 1, 2 and 3 were shown in the appendix.

Table 1. Pathological integral of small intestine of each group of post MTX D1,D3,D5,D7.

Group	Post-MTX D1	Post-MTX D3	Post-MTX D5	Post-MTX D7
A	0	0	0	0
B	6±2	30±3☆	16±2☆	4±2
C		12±2☆★	8±2☆★	
D		19±3☆★	12±4☆	
E		25±4☆	15±3	

Note: D1, D3, D5 referred to the post-MTX injection 1st, 3rd and 5th day. ☆ Compared with the A group, *P*<0.05; ★ compared with the B group, *P*>0.05. Group A: Noral control; Group B: MTX control; Group C: High-dose IL-11 pretreatment before MTX; Group D: High-dose IL-11 treatment after MTX; Group E: Low-dose IL-11 treatment after MTX. At the D3, Group C compared with the group B: P<0.01 (t=4.65); Group D compared with the group B: P<0.05 (t=4.11); Group E compared with the group B: *P*>0.05 (t=2.01); Group C compared with the group E: *P*<0.05 (t=3.45); Group D compared with the group E: *P*>0.05 (t=0.786)

Table 2. Impacts of IL-11 on the proliferation of intestinal crypts cells by the PCNA staining ($\bar{x}$±s)

Group	Ratio of nucleus positive staining		Positive-staining nuclei/crypt	
	Post-MTX D3	Post-MTX D5	Post-MTX D3	Post-MTX D5
B	1.8±0.3	3.8±0.4	0.9±0.5	1.7±0.6
C	12.5±1.1☆	19.5±1.9☆	6.8±0.5☆	9.5±3.2☆
D	11.2±0.9☆†	16.8±2.0☆†	5.9±0.4☆†	10.9±1.1☆†
E	7.9±0.2☆†	6.1±0.6☆†	2.9±0.5	5.6±0.5

Note: The crypts and nuclei of 2 rats were counted, with 10 similar crypts and 400 to 600 nuclei in each rat. ☆ Compared with the B group, *P*<0.05; †compared with the C group, *P*>0.05

Table 3. The crypts depth and villus height of l intestinal and its ratio of each group ($\bar{x}$±s)

Group	Crypt depth (mm)		Villus height (mm)		Crypt depth/villus height	
	D3	D5	D3	D5	D3	D5
A	0.75±0.05	0.80±0.10	0.90±0.06	0.87±0.03	0.83±0.02	0.92±0.03
B	0.10±0.03	0.20±0.04	0.26±0.05	0.50±0.04	0.38±0.01	0.40±0.03
C	0.42±0.02☆	0.65±0.04☆	0.58±0.04☆	0.79±0.03☆	0.72±0.05☆	0.82±0.03☆
D	0.36±0.03☆	0.52±0.05☆†	0.52±0.03☆†	0.70±0.04☆	0.69±0.05☆†	0.74±0.03☆†
E	0.18±0.02†	0.30±0.02†	0.31±0.04†	0.56±0.02†	0.58±0.01†	0.54±0.03†

Note: D3, D5 referred to the post-MTX injection 3rd and 5th day. ☆ Compared with the B group, P<0.05; †compared with the C group, *P*>0.05. the comparison among the E, D and B group, *P*>0.05

As it could be seen from the Tables, the villus heights and crypts depths of the C, D and E group were greater than those of the B group at the same time. The comparison between the C and B Group, on the third day showed that the difference was extremely significant (t=5.32, *P*<0.01).

The comparison between the C and E group also showed that the difference was significant (*P*<0.05).

Results of PCNA immunohistochemical staining

Three days after the IL-11 treatment, the intestinal lacunar cells began to significantly proliferate and proliferated excessively on the 5th day (*P*<0.05).

The ratio of PCNA positive staining nuclei, as well as the absolute numbers of positive staining nuclei inside each crypt were significantly higher than the control group (*P*<0.05).
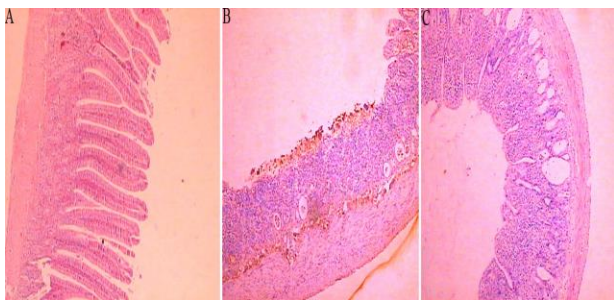
Figure 1. Pathological examination of intestinal tissue: Hematoxylin and eosin (HE) staining
A: Group A (normal control): Wistar rat's small intestinal tissue sections HE 200×; B: Group B (MTX control): Methotrexate (MTX) injection d3 Wistar rat's small intestinal tissue sections HE 200×; (Villi in the small intestine necrosis, inflammatory cell infiltration, crypt abscess). C: Group C (MTX + IL-11 control): D3 MTX injection Wistar rat's small intestinal tissue sections HE 200× (The intestinal villi flat, inflammatory cell infiltration, crypt abscess, mucosal hypertrophy). HE staining. Magnification 200× Scale bar lengths 10 μm. The arrow indicates neutrophils within a necrotic crypt
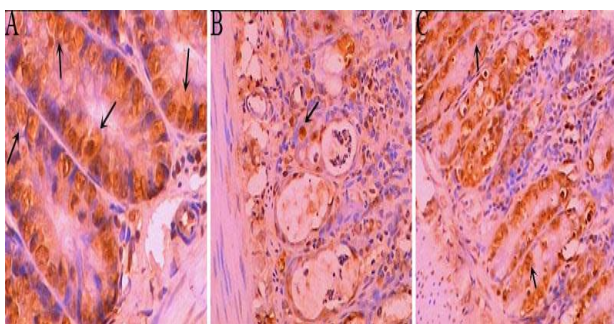
Figure 2. Pathological examination of intestine tissue: immunohistochemical staining of PCNA
A: Group A (normal control): Intestinal crypt cells PCNA immunohistochemical staining 400× (R); B: Group B (Methotrexate (MTX) control): Intestinal crypt cells PCNA immunohistochemical staining 200× (The proliferation of small intestinal crypt cells almost stopped, PCNA staining positive nuclear ratio decreased significantly); C: Group C (MTX + IL-11 control): Intestinal crypt cells PCNA immunohistochemical staining 400× (The proliferation of small intestinal crypt cells was strong, PCNA staining positive nucleus significantly increased the ratio). Magnification 400X. Scale bar lengths 10 μm. The arrows indicate PCNA staining positive nucleus cells
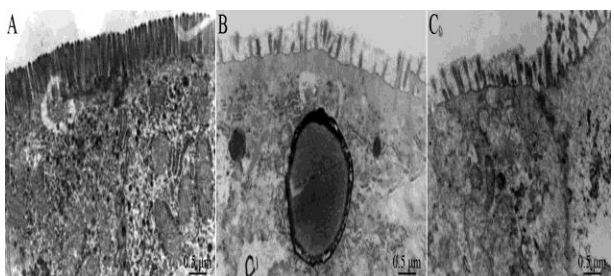
Figure 3. Electron microscopy images
A: Group A (normal control): Normal rat small intestine microvilli free margin 10.0K×; B: Group B (MTX control): Methotrexate (MTX) injection d3 small intestinal tissue free border microvilli 10.0K× (Intestinal epithelial microvilli obviously shorter free margin, sparse, flat); C: Group C (MTX + IL-11 control): MTX injection d3 small intestinal tissue free border microvilli 10.0K× (Intestinal epithelial microvilli increased free margin, growth)

Discussion

It is well known that IL-11 can promote the development and maturation of megakaryocytes. Also, it had been widely used in the treatment of chemotherapy-caused secondary thrombocytopenia. In recent years, it was found that IL-11 also has immunomodulatory, and anti-inflammatory roles as well as epithelial cell-regulatory effects (22). Based on this theory, in this study we established rat intestinal mucositis model, aiming to verify the anti-inflammatory mechanisms and the mucosal protective effects of IL-11 through different doses and different dosing time points. According to the results of this study, HDMTX-induced intestinal mucositis was an inflammatory process of acute injury. Different dosing time points and different doses of IL-11 could reduce the pathological scores of mucositis, with the villus length and lacunar depth significantly increased in comparison with the control group. The proliferation of intestinal lacunar nuclei was accelerated, and the mortality rate was declined, which showed a dose-dependent relationship. In this study, the pathological changes of mucositis after the HDMTX injection were aggravated, and the incidence of mucositis was significantly higher than other reports in the literature (1), which was deduced that it can partly because the eating and drinking were severely affected after the HDMTX injection. This situation was equivalent to the situations of non-hydration and non-alkalization.

IL-11 was a hematopoietic growth factor derived from the bone marrow stromal cells, and played a significant role in the regulation of hematopoiesis, while the mechanisms that how it affected the epithelial cells, thus playing the regulatory roles towards the epithelial cells and the anti-inflammatory effects were still unclear. Through the experiment, we found that the IL-11 pretreatment group and the high-dose group exhibited the faster recovery speed of intestinal mucosa, and the probable reasons might be related with that IL-11 could accelerate the mitosis of intestinal lacunar cells. This feature, which was basically consistent with the previous report, was verified by the PCNA immunohistochemical staining (18). PCNA was mainly synthesized in the G1 and S phase of cell cycle, playing important roles in the cellular proliferation. It has been previously reported that IL-11-accelerated mucosal recovery was because it speeded the proliferation of gastrointestinal mucosal cells and gastrointestinal wall muscle cells (19). It has also been demonstrated that IL-11 might reduce the expressions of pro-inflammatory factors, such as TNF-αs (20, 22). Findings have shown that transient cell cycle arrest is a possible mechanism by which rhIL-11 may protect intestinal epithelial cells from damage induced by chemotherapy or radiation therapy (23). In brief, in-depth studies are still needed to reveal the anti-infective mechanisms of IL-11. The results of

in vivo studies on rat model of mucositis indicated that IL-11 might act through directly penetrating the epithelial cells and tissues.

In this study, the human T-lymphoblastic leukemia cell line (CEM) was used as the target, and morphological observation was also used to investigate the impacts of different -concentration of IL-11 towards the CEM proliferation and the anti-tumor effects of MTX. Findings showed that IL-11 could not promote the proliferation of CEM cells, thus it could be safely used for the bone marrow suppression after the HDMTX chemotherapy.

Conclusion

IL-11 could reduce the severity of HDMTX-induced intestinal mucositis, and improve the survival rate of experimental rats. But, IL-11 could not promote the proliferation of CEM cells. IL-11 could be safely used as the adjuvant treatment of HDMTX in childhood leukemia.

Acknowledgment

Conflict of interest

All authors have no conflict of interest regarding this paper.

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