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Treatment of chemotherapy-induced leukopenia in a rat model with aqueous extract from *Uncaria tomentosa*

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Summary

The Uncaria tomentosa water extracts (C-Med-100®) depleted of indole alkaloids (< 0.05%, w/w) have been shown to induce apoptosis and inhibit proliferation in tumor cells in vitro and to enhance DNA repair, mitogenic response and white blood cells in vivo. In this study, the effect of C-Med-100® in the treatment of chemically induced leukopenia was evaluated in a rat model. W/Fu rats were treated first with doxorubicin (DXR) 2 mg/kg x 3 (i.p. injection at 24 hour-intervals) to induce leukopenia. Twenty-four hours after the last DXR treatment, the rats were daily gavaged with C-Med-100® for 16 consecutive days. As a positive control, Neupogen®, a granulocyte colony stimulator was also administered by subcutaneous injection at a dose of 5 and 10 µg/ml for 10 consecutive days. The results showed that both C-Med-100® and Neupogen® treatment groups recovered significantly sooner ($p < 0.05$ by Duncan test) than DXR group. However, the recovery by C-Med-100® treatment was a more natural process than Neupogen® because all fractions of white blood cells were proportionally increased while Neupogen® mainly elevated the neutrophil cells. These results were also confirmed by microscopic examination of the blood smears. The mechanism of the C-Med-100® effect on WBC is not known but other data showing enhanced effects on DNA repair and immune cell proliferative response support a general immune enhancement.

Key words: Uncaria tomentosa, rat, Doxorubicin, leukopenia, DNA repair

Introduction

Chemotherapeutic agents are often drugs having the narrowest therapeutic indices in all medicine. The therapeutic dose is often restricted by the nonselective toxic effects on normal tissues. Hematologic toxicity is the most dangerous form of toxicity for most of the antineoplastic drugs used in clinical practice. The most common form is neutropenia, with an attendant high risk of infection. Chemotherapy can also induce qualitative defects in the function of both polymorphonuclear leukocytes and platelets, further aggravating the clinical impact of bone marrow suppression. Most of the commonly used antineoplastic agents are also capable of suppressing both cellular and humoral immunity, and predispose patients to various complications from infection (Maxwell and Maher 1992; Kim and Demetri 1996; Bow 1998).

The basic understanding of neutrophil biology and the physiology of chemotherapy-induced neutropenia have advanced tremendously in the past two decades. Pharmacologic interventions capable of reducing or preventing side effects could have a substantial impact on cancer management (Trotti 1997). Adjunctive cytokine and progenitor cell support of hematologic recovery after myelosuppressive therapy have proved to be models of translational research and have led to

Abbreviations
DMSO – dimethyl sulphoxide; DSB – (DNA) double strand breaks; DXR – doxorubicin, i.e. adriamycin; FCS – fetal calf serum; G-CSF – granulocyte colony stimulating factor; GM-CSF – granulocyte macrophage colony stimulating factor; NAD – nicotinamide adenine dinucleotide; PHA – phytohemagglutinin; SCID – severe combined immune deficiency; SSB – (DNA) single strand breaks; WBC – white blood cells.

novel therapeutic initiatives for patients with cancer and hematologic malignancies. The development of the clinical use of granulocyte, or granulocyte/macrophage colony stimulating factors (G-CSF, GM-CSF) has made it possible to test the effectiveness of escalated doses of chemotherapy in patients with cancer because they can accelerate granulocyte recovery and shorten the time between chemotherapy cycles which also can lead to intensification of the chemotherapeutic dose (Hornedo & Cortes-Funes 1996; Johnston and Crawford 1998; Gilmore et al 1999).

Uncaria tomentosa, commonly known as cat's claw or "una de gato", is used in traditional Peruvian medicine for the treatment of arthritis, inflammations, cancer, allergy, and viral infections (Jones 1995). The extracts or components of this plant have been shown to have anti-inflammatory, antiviral, antimutagenic, antioxidant activities as well as to have an enhancement of phagocytosis (Keplinger 1982; Wagner et al 1985; Cerri et al 1988; Aquino et al. 1989; 1991; Rizzi et al. 1993; Desmarchelier et al. 1997; Wurm et al. 1998; Sandoval-Chacon et al. 1998). Previous studies have shown a proprietary aqueous extract from Uncaria tomentosa (C-Med-100®) to be an effective inducer of apoptosis in vitro (Sheng et al. 1998) and en enhancer of DNA repair and immune response in vivo (Sheng et al. 2000). Both rats and human volunteers supplemented with C-Med-100® were also observed to have an increased tendency for elevation of WBC. This fact has inspired us to investigate the possibility of using C-Med-100® to treat chemotherapy-induced leukopenia in a rat model.

• *Apparatus*

HPLC:	AS-2000A Autosampler (Merck-Hitachi), L-6200A Intelligent Pump (Merck-Hitachi) L-4500A Diode Array Detector (Merck-Hitachi), Oven (Merck-Hitachi)
Software:	D-7000 Chromatography Data Station Software 3.1.1
Column:	LiChrospher 100 RP18 (125 mm x 4 mm) (Merck)
Precolumn:	LiChrospher 100 RP18 (4 mm x 4 mm) (Merck)

• *Conditions*

Solvents:	A: buffer (solution 1: KH_2PO_4 1.36 g / l; solution 2: 1,42 g / l; 643 ml of solution 1 mixed with 347 ml of solution 2) B; methanol / acetonitrile (1 : 1)
Gradient:	40 % B to 65 % B in 30 min.
Oventemp.:	15°C
Flow:	1.0 ml / min.
Detektion:	245 nm

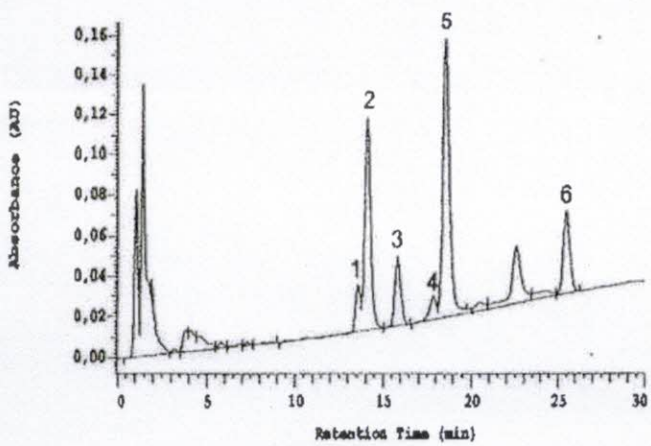

Fig. 1. HPLC-fingerprint analysis.

Materials and Methods

Materials

Uncaria tomentosa extract was supplied commercially as C-Med-100® dried powder extract by CampaMed, Inc. (New York, NY, USA). C-Med-100® is a 90–100 °C hot water extract of Uncaria tomentosa containing only molecules < 10, 000 molecular weight (MW). The active ingredients of C-Med-100® are > 8% carbonyl alkyl esters depleted of large molecular weight conjugates (> 10,000 MW) and of indole alkaloids (< 0.05%, w/w, see HPLC-fingerprint analysis). The pale-yellow powder was dissolved directly in sterile tap water for oral administration to the animals at doses of 40 and 80 mg/kg. Doxorubicin (DXR, i.e. adriamycin) was commercially available from Pharmacia & Upjohn and it was administered intraperitoneally to rats at a dose of 2 mg/kg x 3 with 24-hour intervals. Granulocyte colony stimulating factor Neupogen® (0.3 mg/ml) was commercially available from Roche (Grenzach-Wyhlen, Gemany) and it was given by subcutaneous injection to rats as a positive control to C-Med-100® at the dose of 5 and 10 µg/kg/day for 10 consecutive days.

Fingerprint analysis and alkaloid quantification of Uncaria tomentosa extract C-Med 100®

(according to Sturm and Stuppner, 1992)

• *Sample preparation*

To 315 mg C-Med 100 (LOT-No. E-40639) 10ml 2 % sulphuric acid-solution was added and mixed for 5 min. in ultrasonic bath. The mixture was extracted three times with 10 ml ethylacetate each. The aqueous phase was separated and adjusted to pH 10 with ammonia solution and then extracted four times with 10 ml of ethylacetate each. The organic extracts were combined, evaporated to dryness and the residue dissolved in 0.5 ml of methanol.

• *Content of alkaloids*
in 100g Uncaria tomentosa extract C-Med 100® (mg):

Peak No.	Alcaloid	C-Med 100®
1	Uncarin F	2.39
2	Speciophyllin	13.75
3	Mitraphyllin	4.34
4	Isomitraphyllin	1.73
5	Pteropodin	20.17
6	Isopteropodin	5.96

The total amount of the main alkaloids of C-Med 100® yielded ~ 0,05 % of the extract.

Animals

Female Wistar-Furth rats, weighing 150–200 grams, were housed 2–3 to a cage at ambient temperature of 21 to 23°C. The lights were on from 6 a.m. to 6 p.m. and the rats had free access to fresh tap water and standard pellet food. The rats were randomly assigned into different groups and there was no statistically significant difference among the groups in body weight and white blood cells (WBC) at the beginning of each experiment. The body weight of individual animals and the food consumption of each cage (2–3 rats) were recorded every week. At the end of each experiment, animals were sacrificed and the heart, liver, spleen, kidney and thymus tissues were weighed and collected in phosphate-buffered formalin solution for pathological examination. Half of the spleen was processed to single cell suspension in ice-cold homogenizing buffer (15 mM Tris, 60 mM NaCl, 0.34 M sucrose, 10 mM 2-mercaptoethanol and 10 mM EDTA, pH 7.4) and DMSO was added for storage at -80°C until the assay of DNA strand breaks by alkaline elution. The animals were treated according to the Swedish guidelines for humane treatment of laboratory animals. The experiments were approved by the Ethical Committee at the University Hospital in Lund, Sweden.

Alkaline elution

Frozen spleen single cell suspensions were rapidly thawed at 37°C and layered directly onto 25 mm diameter and 2 µm pore size polycarbonate filters (Millipore). DNA single strand breaks were measured by alkaline elution as described by Kohn et al (Kohn et al. 1981) with modifications (Olsson et al. 1996) to measure the unlabeled DNA by microfluorometry (Cesarone et al. 1979).

Hemotologic parameter

The blood samples were collected into heparin tubes and then analyzed within one hour by an automated hematology analyzer (Sysmex, K-1000). A control blood sample (ECN-13, Sysmex™) was routinely checked for the quality control.

Morphological evaluation of the leukocyte fractions

Peripheral blood smears were collected at day 0, day 10, day 15 and day 22 after Doxorubicin treatments. The smears were stained by May-Grünewald-Giemsa (MGG) staining and then evaluated by a trained technician with coded slides for counting. Differential morphological counting of blood smears was used to compare and evaluate the types of WBC determined by automated blood analysis (Sysmex).

Histopathological Examination

When the rats were sacrificed liver, kidney, spleen and heart tissues were immersion-fixed in phosphate-buffered formalin. The tissues were embedded in paraffin and 5–6 µm sections were stained with hematoxylin-eosin. All slides were evaluated by light microscopy.

Statistics: Comparison of mean differences among groups was made by analysis of variance with further analysis between two groups by Duncan test at a significant level of $p \leq 0.05$. The relationship between the two lymphocyte counts was analyzed by Pearson correlation.

Results

Toxicity

The body weight growth curves after doxorubicin and C-Med-100® treatments are shown in Figure 2. Com-

Table 1. Organ weight coefficients (=organ weight/body weight x 100) in doxorubicin (DXR) treated rats with or without C-Med-100 supplement.

Group	N	Spleen	Heart	Kidneys	Liver
Control	11	0.210 ± 0.009	0.373 ± 0.028	0.730 ± 0.029	3.707 ± 0.222
DXR (2 mg/kg, 3 x i.p.)	12	0.208 ± 0.016	0.369 ± 0.022	0.729 ± 0.032	3.706 ± 0.196
DXR + CMed100 (40 mg/kg)	12	0.210 ± 0.013	0.380 ± 0.023	0.734 ± 0.033	3.778 ± 0.158
DXR + CMed100 (80 mg/kg)	12	0.214 ± 0.012	0.386 ± 0.034*	0.725 ± 0.018	3.878 ± 0.207

* $p < 0.05$ compared to DXR group by Duncan test.

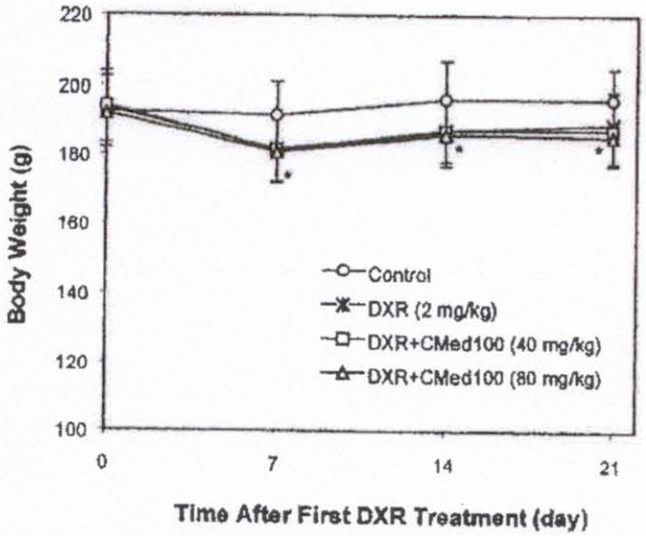

Fig. 2. Body weight change after drug treatments. W/Fu rats were given DXR by ip injection at 2 mg/kg x 3 at 24-hour intervals. Daily C-Med-100® gavage (40 and 80 mg/kg) began 24 hours after the last DXR injection. Results are shown as mean and SD (point and error bar) with n = 11–12. * indicates $p < 0.05$ for DXR treated groups compared to control group by Duncan test.

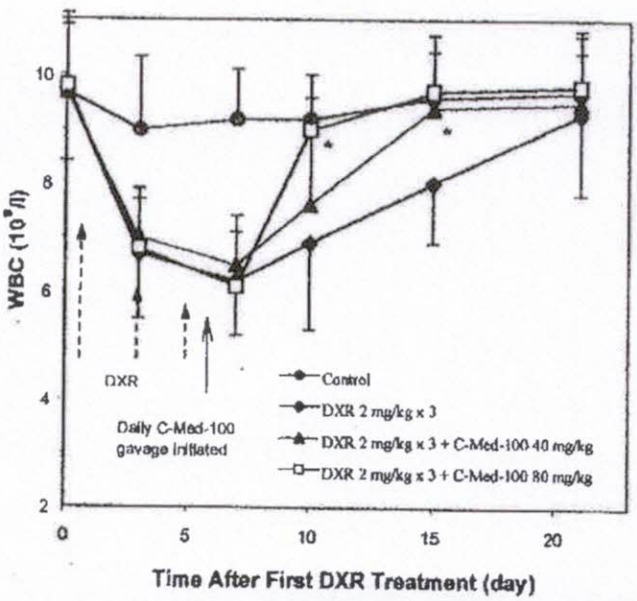

Fig. 3. C-Med-100® treatment of DXR induced leukopenia in a rat model. W/Fu rats were first treated with DXR at 2 mg/kg x 3 (Monday, Wednesday and Friday) and the C-Med-100® treatment initiated 24 hours after the last treatment of DXR. Blood was sampled from rat orbital vessels in heparinized tubes at the indicated time points and analyzed by an automated hematological analyzer for WBC content. Results shown are mean ± SD (n = 11–12). * indicates statistically significant difference by Duncan test when compared to DXR treatment (2 mg/kg x 3).

pared with controls, doxorubicin treatment groups with or without C-Med-100® in combination, showed statistically significant reduction in body weight (about 5 %) one week after the first DXR treatment. The differences were present throughout the experiment ($p < 0.05$ by Duncan test). No differences were found between doxorubicin or doxorubicin plus C-Med-100® treatment ($p > 0.05$ by Duncan test). These results suggested the toxicity be directly related to DXR but not to C-Med-100® treatment. Table 1 showed the organ weight data obtained from this experiment expressed as organ coefficient; i.e. individual organ weight divided by the individual body weight as a percentage. There was no significant difference among the DXR treated groups with or without C-Med-100® supplements (40 and 80 mg/kg) in spleen, kidney and liver organ coefficients. Although there was a significant difference ($p < 0.05$ by Duncan test) in heart coefficient between DXR treatment group and C-Med-100 supplement group at 80 mg/kg, there were no significant differences when these two groups were compared with controls ($p > 0.05$ by Duncan test).

Treatment of Leukopenia

The result of C-Med-100® treatment on DXR-induced leukopenia is shown in Figure 3. As indicated in the graph, DXR at the dose of 2 mg/kg x 3 i.p. injections induced a rapid drop in WBC after the second DXR treatment and continued to decrease after the third treatment. However, C-Med-100® daily treatments (40 and 80 mg/kg) initiated 24 hours after the last DXR treatment recovered the WBC significantly sooner than the DXR treatment only group in a dose dependent manner ($p < 0.05$ by Duncan test). The effect on WBC of C-Med-100® + DXR treatment was also compared with Neupogen® (G-CSF) at the doses of 5 and 10 μg/kg (data not shown). Neupogen® recovered WBC rapidly in about 24 hours and sometimes the WBC were even higher than the normal level. Neupogen® was also shown to increase only the non-lymphocyte fractions of WBC having insignificant effect on the mononuclear leukocyte fractions (Figure 4). Although C-Med-100® and Neupogen® both stimulated rapid recovery of WBC, the recovery by C-Med-100® was more of a natural process since all fractions of WBC increased proportionally compared to Neupogen® (Figure 4). In order to confirm this finding subgroups of animals were randomly chosen, and blood smear samples were prepared for morphological examination by light microscopy. The results were compared with those obtained by blood cell automated analysis from the same sample. There was a very close relationship between the results of Sysmex automatic analysis and blood smears (Pearson correlation coefficient $r = 0.9389$, $n = 15$, $p < 0.01$; Figure 5).

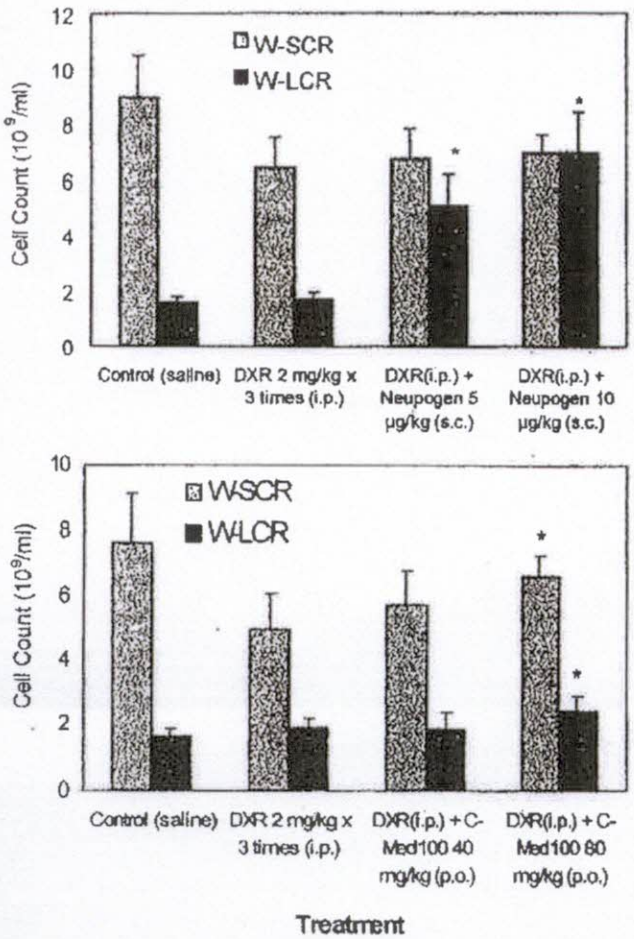

Fig. 4. Comparison of C-Med-100® and Neupogen® treatments on the change of rat leukocyte fractions. W/Fu rats were first treated with doxorubicin at the dose of 2 mg/kg x 3 times (Monday, Wednesday and Friday) and C-Med-100® (40 mg/kg and 80 mg/kg by gavage daily) or Neupogen® (5 µg/kg and 10 µg/kg, s.c., 10 times) treatments were initiated 24 hours after the last DXR treatment. Blood samples were taken on day 10 after the first injection of Doxorubicin and analyzed by an automated haematological analyzer. W-SCR: white blood cells with small size (i.e. lymphocyte rich fraction; W-LCR, white blood cells with larger size (i.e. neutrophil rich fraction). Data were shown as mean ± SD (n > = 10) * indicated statistically significant difference when compared to DXR treatment ($p < 0.05$ by Duncan test).

DNA repair: On day 10 after the first DXR injection, a separate subgroup of rats (n = 4 for each group) were sacrificed and the spleen single cell suspensions were prepared and DNA single strand breaks were evaluated by alkaline elution. The results are presented in Figure 5 comparing DXR treated rats with and without C-Med-100® supplementation. Rats supplemented with C-Med-100® at the daily dose of 80 mg/kg showed a significant increase ($p < 0.01$ by Duncan test) of DNA repair while rats with 40 mg/kg did not differ from the group of DXR alone.

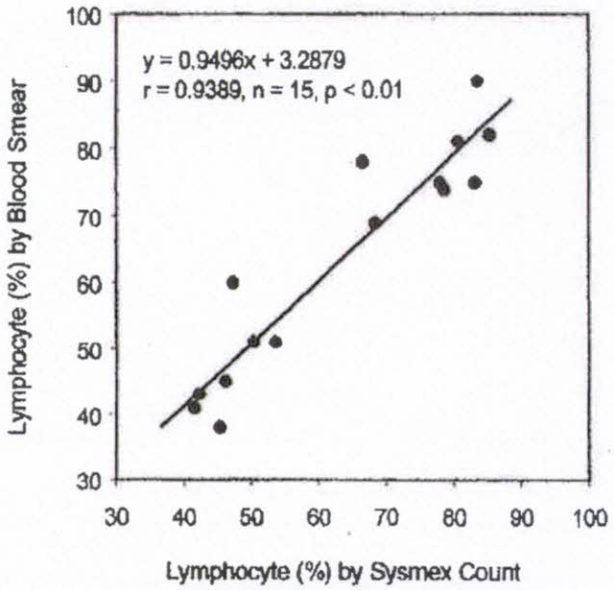

Fig. 5. The lymphocyte fraction count (%) determined by Sysmex automated hemotological analysis is correlated to blood smear examination by light microscope on 15 rat blood samples on day 10 after the first DXR treatment. There was a very strong correlation (Pearson correlation coefficient r = 0.95, $p<0.01$) between the two methods.

Discussion

DXR is an anthracycline, which is a class of drugs widely used as effective antineoplastic drugs. DXR is toxic to hematopoietic precursor cells and it can produce immediate and prolonged bone marrow depressions. The classical dose-limiting adverse effects are acute myelosuppression and cumulative dose-related cardiotoxicity (Hortobagyi 1997; Muller et al. 1998). In this study, DXR administered at 2 mg/kg x 3 with 24 hour-intervals produced 5 % drop in body weight growth and about 50 % reduction of WBC within the first week of treatment. The WBC would normally recover in 3 weeks if no further treatment of DXR were given. However, if C-Med-100® or Neupogen® were given as a treatment, the recovered course of WBC depletion was significantly shortened (Figure 3). More interestingly unlike Neupogen®, which mainly recovered non-lymphocyte fractions, C-Med-100® recovered all fractions of WBC in a proportional way which are closer to the natural process (Figure 4). This supports that C-Med-100® has a different mechanism of action for overcoming chemotherapeutic induced leukopenia. The proportional elevation of all fractions of WBC by

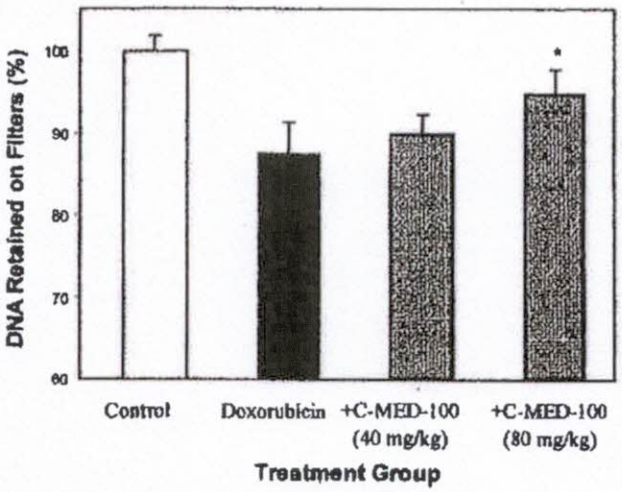

Fig. 6. DNA repair in W/Fu rats treated with DXR (2 mg/kg x 3 i.p. injections) ± C-Med-100® supplementation (daily gavage initiated 24 hr after the last DXR treatment) at day 10 after the first DXR treatment. DNA damage and repair were measured by alkaline elution of spleen single cell suspension from female W/Fu rats. Data are shown the averages in column and SD by error bar (n = 4 in each group). * indicates $p < 0.05$ by Duncan test.

C-Med-100® suggests that it may have a direct myelostimulating effect while the fast action of Neupogen® is known to shorten the time to release of newly formed neutrophils (from approximately 5 days to 1 day) (Lord et al., 1989; 1991). Jimenez et al (1992) found ImuVert, a biological response modifier derived from the bacterium Serratia marcescens, could protect Cytoxan or Adriamycin induced neutropenia in adult rats. They concluded that stimulation of endogenous cytokine production by ImuVert might provide a potentially useful approach to bone marrow rescue from chemotherapy. C-Med-100® may well share the same mechanism although much work needs to be done to confirm this. The fact that PHA stimulated lymphocyte proliferation was significantly elevated in splenocytes of rats treated with C-Med-100® at the doses of 40 and 80 mg/kg (Sheng et al., 1999), together with a recent study that Uncaria tomentosa induced human endothelial cells to release a lymphocyte-proliferation regulating factor (Wurm et al., 1998), further supports a cytokine mediated myeloproliferative effect.

The other mechanism of C-Med-100® may be related to its DNA repair enhancement effects. Anthracyclines have been known to inhibit topoisomerase II, which occurs as a result of anthracycline intercalation between adjacent DNA base pairs. Production of hydroxyl free radicals is associated with antitumor effects and toxicity to normal tissues (Gewirtz, 1999; Ravid et al., 1999). Watering and associates (1974) reported the inhibition of enzymatic repair of radiation-induced DNA single-strand breaks by doxorubicin. Adriamycin-induced DNA single strand breaks at 1 μg/ml were completely repaired within 48 h after drug removal in both human fibroblasts (CLV98) and human melanoma cells (ME18). However, no repair of single strand breaks was observed in ME18 and CLV98 cells after drug treatment at the concentration of 5 μg/ml (Anuszewska and Gruber 1994). In cells with low catalase activity no repair of adriamycin induced lesions was observed up 48 h post treatment. In cells with high catalase activity after 48 h the repair was either complete or partial depending on the human or mouse cell type used (Gruber et al. 1998). The previous studies have shown that DNA repair of single strand breaks (SSB) and double strand breaks (DSB) in splenocytes 3 hours after 12 Gy whole body irradiation of rats were significantly improved with C-Med-100® treatment for 8 weeks (Sheng et al. 1999). Although DNA damage was induced by DXR and the treatment of C-Med-100® was only for about 2 weeks, the DNA repair of single strand breaks in splenocytes was significantly enhanced compared to the DXR treatment group ($p < 0.05$ by Duncan test). These data clearly demonstrate the potential health benefit of C-Med-100® for the treatment of chemotherapy-induced leukopenia, because not only the WBC recover faster (Figure 3) but they function better in their cellular responses to DNA damage (Figure 6).

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