Action of melatonin on bone marrow depression induced by cyclophosphamide in acute toxicity phase

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Abstract

BACKGROUND: In the course of the last few years, various studies have researched the relations linking melatonin (MLT) with hematopoiesis and the immune-hematopoietic system. Nevertheless, to date there are numerous issues still opened and many questions are yet unanswered. Much emphasis has been recently placed on the reducing role of MLT, which has been demonstrated by many studies to mitigate the genotoxic damage inflicted by various alkylating agents. Although *in vitro* MLT has shown to be effective in limiting the cytological alterations provoked by chemotherapeutic drugs, some clinical studies seem to point to a somewhat lower effectiveness in countering chemotherapy-induced leukopenia and anemia.

OBJECTIVE: The aim of this study was to evaluate the activity exerted by pharmacological doses of MLT in limiting leukopenia, anemia and other hemocytometric modifications in animals facing acute toxicity caused by the treatment with cyclophosphamide (CP). Moreover, we have compared the activity of MLT with well-known alpha-tocopherol/ascorbic acid antioxidant system.

RESULTS: Our results indicate that overall MLT exerts a remarkable countering activity towards leukopenia and anemia in the early phase of administration of CP.

CONCLUSIONS: Our findings suggest possible active involvement of MLT in hematopoiesis and erythrocyte- and leukocyte turnover. This paper summarizes the essential aspects of the available literature, researching the possible relations between MLT and immune-hematopoietic system.

Abbreviations :

MLT	- melatonin
CP	- cyclophosphamide
DMSO	- dimethylsulphoxide
BM	- bone marrow
CFU-GM	- colony forming units for granulocytes and macrophages
GPCR	- G-protein coupled receptors
APUD	- Amine Precursor Uptake Decarboxylase

INTRODUCTION

Our laboratory has been studying for a long time existing relations between N-acetyl-5-methoxytryptamine or melatonin (MLT), hematopoiesis, blood physiology and the immune system (Di Bella *et al.* 1969; Di Bella *et al.* 1979, a). These studies, begun in the mid '60s by Di Bella and colleagues focused mainly on the effects of MLT on red bone marrow (BM) (Di Bella *et al.* 1976), thrombopoiesis (Di Bella *et al.* 1979, b; Rossi *et al.* 1988), platelet aggregation (Di Bella *et al.* 1980), erythropoiesis (Di Bella *et al.* 1979, a) and lymph circulation (Rossi *et al.* 1974; Di Bella & Gualano, 2006). Subsequent contributions have been made by various authors (Haldar *et al.* 1992, a; Maestroni, 1998; Maestroni, 1999; Labunets *et al.* 2004) on the existence of a link between MLT, hematopoiesis, functions of blood and immunocompetent cells. However, despite intensive research done in this area, there are still many aspects which need to be clarified concerning the role of MLT in hematopoiesis and blood physiology.

MLT is the main product of the pineal gland or epiphysis. It is well known that its maximum secretion and plasmatic peak is reached during the darkness of night. However, in recent years, studies have highlighted its presence and its *de novo* synthesis also in extrapineal organs (Kvetnoy, 1999), including hematopoietic and lymphoid organs, the BM and the thymus (Conti *et al.* 2000; Naranjo *et al.* 2007), and the hematopoietic and differentiated immunocompetent cells, i.e. mononuclear phagocytes, natural killer cells, eosinophils, platelets and, according to some evidence, even red blood cells (Rosengarten *et al.* 1972; Launay *et al.* 1982; Finocchiaro *et al.* 1988; Champier *et al.* 1997; Tan *et al.* 1999; Carrillo-Vico *et al.* 2004; Morera & Abreu, 2005). MLT is also said to be present in organs unrelated to the hematopoietic, immunocompetent and hematologic system, like retina, ovaries and various other organs (Kvetnoy *et al.* 1999; Jaworek *et al.* 2007; Konturek *et al.* 2007). It is also synthesised in the enterochromaffin cells (Raikhlin & Kvetnoy, 1976), in cells belonging to the APUD system and even in structures not belonging to the diffuse neuroendocrine system, so much so that some authors question the real belonging of MLT to the endocrine system in the classic, strict sense of the word. Moreover, the concentrations of extrapineal MLT are not always correlated to the sleep/wake cycle: for example, despite most plasmatic MLT deriving from pineal secretion, in platelets it is synthesised and accumulated independently of the photoperiod (Reiter & Tan, 2003; Morera & Abreu, 2005; Jaworek *et al.* 2007; Konturek *et al.* 2007).

Because of the relatively high concentrations of MLT in the BM (Tan *et al.* 1999), and in many hematopoietic and immunocompetent cells, the hypothesis of a real and active involvement of MLT in hematopoiesis and in immune hematological processes seems to be at least plausible. MLT present in the BM, mononu-

clear phagocytes, platelets, T lymphocytes, thymus and other lymphoid organs appears to be produced and synthesised *in situ* (Rosengarten *et al.* 1972; Launay *et al.* 1982; Finocchiaro *et al.* 1988; Champier *et al.* 1997; Tan *et al.* 1999; Conti *et al.* 2000; Carrillo-Vico *et al.* 2004; Morera & Abreu, 2005; Naranjo *et al.* 2007). Therefore it seems possible that MLT may also act as an autocrine/paracrine factor in the physiology and biochemical dynamics of the BM and of the immune-hematopoietic system, the hypothesis presented also by other authors (Carrillo-Vico *et al.* 2004; Markus *et al.* 2007). This possibility is further supported by the fact that many cells of this system, including platelets and T lymphocytes, synthesise MLT and express its receptors (Vacas *et al.* 1972; Di Bella *et al.* 1979, c; Pozo *et al.* 1997; Drazen *et al.* 2001; Yau *et al.* 2002; Regodón *et al.* 2009). Similarly, in many extra-pineal locations in which it is synthesised, it is supposed to acts as a local factor. Despite numerous studies performed on differentiated blood cells and documentation of an independent immune regulation (Carrillo-Vico *et al.* 2005; Szczepanik, 2007), platelet antiaggregation (Di Bella *et al.* 1979, d; Leach & Thorburn, 1980; Kornblihtt *et al.* 1983; Cardinali *et al.* 1993; Di Bella & Gualano, 2006), and protective effect on the erythrocyte membrane (Tesoriere *et al.* 1999; Kedziora-Kornatowska *et al.* 2007), a lot of information still needs to be uncovered about its role in hematopoiesis and in differentiative processes.

MLT has been reported to be useful in the stimulation and regulation of immune-hematopoietic processes: in preliminary clinical trials it has shown a marked tropism for the hematopoietic system, revealing the capacity to attenuate the extent of thrombocytopenia in patients undergoing chemotherapy and radiotherapy (Lissoni *et al.* 1995, a; Lissoni *et al.* 1995, b; Lissoni *et al.* 1996; Lissoni, 2007; Todisco, 2007), and to act directly on the immune responses by stimulating the CD4+ T helper cells (Maestroni, 1995), which express MLT receptors (Pozo *et al.* 1997; Drazen *et al.* 2001; Carrillo-Vico *et al.* 2003). It has been proven that MLT may significantly influence the pool of cytokines secreted, which in turn influence the activity of helper T cells of both the Th1 and Th2 subtypes (Maestroni, 1995; Liu *et al.* 2001; Carrillo-Vico *et al.* 2003). Although its effects on the immune system appear to be particularly complex and in some instances opposed (Majewska *et al.* 2007), it seems that MLT may also act as a stimulant of cell-mediated immunity, showing a specific modulatory activity on the immune system (Maestroni, 1995; Liu *et al.* 2001; Santello *et al.* 2008). It is also interesting to note the MLT capability of *in vitro* regulation of the proliferation of various lines of lymphoblasts, both normal and transformed, inhibiting the incorporation of tritiated thymidine (Persengiev & Kyurkchiev, 1993). MLT is also active in the inhibition of proliferation of human B lymphoma cells and other tumours of the hematopoietic system (Yu *et al.* 2000; Trubiani *et al.* 2005; Rubio *et al.* 2007; Bejarano *et al.* 2009).

Overall, an immune stimulating effect of MLT has been widely reported, suggesting its use in patients suffering from acquired immune deficiency (Lissoni *et al.* 1995, c; Kast & Altschuler, 2006). It is interesting to note how this stimulation of the immune system has been connected with the genesis of various autoimmune diseases (Sandyk, 1993). Research on heterologous transplants in rats and in subjects affected by rheumatoid arthritis and other immune disorders (Kang *et al.* 2001; Jung *et al.* 2004; Cardell *et al.* 2008; Korkmaz, 2008) suggests that MLT, rather than a generic stimulating influence on immune processes, has a real and direct regulating and modulating role of the immune response: *in vitro* studies showed that MLT is efficacious in the inhibition of proliferation and the induction of apoptosis in human lymphoma cell lines (Trubiani *et al.* 2005; Bejarano *et al.* 2009), as well as useful in individuals suffering from myelodysplasia (Viviani *et al.* 1990). It should be noted that such effects have been ascribed to the induction or the block of apoptosis. Since the latter plays a central role in immunological and hematopoietic processes (Cacciapaglia *et al.* 2009; Droin *et al.* 2009), and since MLT has been widely proven to be able to modulate the apoptotic process in a biphasic mode (Lissoni *et al.* 1995, c; Mayo *et al.* 1998; Yu *et al.* 2000; Scott *et al.* 2001; Trubiani *et al.* 2005; Cheng *et al.* 2006; Feng *et al.* 2006; Rubio *et al.* 2007; Bejarano *et al.* 2009), it cannot be excluded that in addition to its role in the secretion of cytokines (Liu *et al.* 2001) and in endocrine secretion (Mauri *et al.* 1985; Lissoni *et al.* 1987; Aleandri *et al.* 1996; Baltaci *et al.* 2004; Mogulkoc *et al.* 2006), it may also act directly by modulating the apoptotic process during immune responses and hematopoiesis. Such effects appear to rely on its physiological or pharmacological concentrations, and the manner in which the substance is administered.

Moreover some evidence suggests benefit effect of MLT in reduction of anaemia and hemoglobin deficiency in elderly patients chronically treated with MLT (Herrera *et al.* 2001; Hörl, 2002; Labonia *et al.* 2005; Arushanian *et al.* 2006). In mice and Wistar rats MLT has shown to be particularly active in reducing damage to the BM induced by various chemicals and genotoxic agents (Melchiorri *et al.* 1998; Rapozzi *et al.* 1998). MLT showed to be active in reducing cytotoxic damage to the BM in mice after irradiation with gamma rays (Vijayalaxmi *et al.* 1999), but also in increasing the number of circulating blood cells in rats (Koc *et al.* 2002), suggesting that it may act as a regulatory and stimulating factor on macrophages and granulocytes (CUF-GM), thereby fully confirming the hypothesis previously proposed by Haldar, Häussler and Gupta (1992, a; 1992, b). Recently, a similar effect has been suggested by Akbulut *et al.* (1999), while Sutherland *et al.* (2002) have pointed out a possible direct role of MLT in leukocytopoiesis and in the release of white blood cells into circulation.

The reducing properties of MLT has been well documented for years, showing it to be an excellent antioxidant even though the precise mechanism has not yet been fully understood. It seems plausible that this involves the oxidation of carbon 2 on the indolic nucleus with formation of a carbocationic intermediate and the opening of the indolic nucleus with the subsequent formation of kynurenic compounds (Hirata *et al.* 1974; Reiter *et al.* 1999), which also have reducing capability (Goda *et al.* 1999). Widespread research has demonstrated this important antioxidant activity (Sandyk, 1990; Ianăș *et al.* 1991; Pieri *et al.* 1994; Acuña-Castroviejo *et al.* 2001; Reiter *et al.* 2001, a; Martin *et al.* 2002; Baydas *et al.* 2003; Kaptanoglu *et al.* 2003; Leaden & Catalá, 2007), mainly because MLT appears particularly active against hydroxyl radicals, a chemical species particularly injurious to cellular functions and implicated in many physiopathological processes (Poeggeler *et al.* 1993; Brömme *et al.* 2000). Moreover, it has been highlighted the action of MLT in the regulation of gene expression and activity of many antioxidant enzymes (Mayo *et al.* 2002).

MLT is widely expressed throughout the world of living organisms, from animals to plants, whether metazoans or non-metazoans (Korf, 1994; Shedpure & Pati, 1995; Hardeland, 1999; Reiter *et al.* 2001, b; Falcón *et al.* 2009), probably thanks to its marked reducing power, a characteristic which has most likely contributed to its evolutionary success. For this powerful antioxidant role, a finalistic hypothesis has been advanced according to which the high concentration of MLT found in the BM is proposed to have the function of oxidative stress reduction in a metabolically highly active tissue subjected to an extremely high cellular turnover (Tan *et al.* 1999). Thanks to its reducing action it is thought to be able to regulate peroxidase activity of neutrophil and eosinophil granulocytes (Allegra *et al.* 2001; Lu *et al.* 2008).

Notwithstanding this clear and very important role in limiting cellular oxidation, many studies have emphasised the presence of specific MLT receptors, both belonging to the family of G-protein coupled receptors (GPCR) and to that of the nuclear receptors within cells of the immune-hematopoietic system. It should be noted that these receptors are present in platelets, T helper lymphocytes, macrophages, the spleen and thymus (Vacas *et al.* 1992; Pozo *et al.* 1997; Drazen *et al.* 2001; Liu *et al.* 2001; Regodón *et al.* 2009). It is therefore very likely that MLT receptors are present in the differentiated compartment of the immune-hematopoietic system.

In our laboratory we demonstrated and documented the activity of MLT in modulating and attenuating the outward K$^+$ currents across the megakaryocyte membrane (Di Bella *et al.* 2002). Other evidence suggests its role in many electric transduction processes, both through the direct effect of MLT on ionic channels and through actions mediated by the activation of the

GPCR receptors. It is interesting to note how Mel1 MLT receptor forms dimers with potassium channels at the CNS level (Huan *et al.* 2001; Zhou *et al.* 2003; Hu *et al.* 2005), while in lymphocyte MLT binds to Kv1.3 potassium channels leading to inactivation of ionic currents (Varga *et al.* 2001). In recent years several studies have highlighted the importance of electrophysiological phenomena in the differentiating processes of cells in the immune-hematopoietic system. It cannot be ruled out that amongst its multiple signalling modes there is also an important electrophysiological mechanism.

In the light of these multiple physiological implications, one can easily imagine the difficulties still faced today in understanding the various activities of MLT. Its ubiquitous presence in the organs and systems suggests its dual mode of signalling and acting: systemic on the one hand, because of its pineal secretion, and on the other hand autocrine/paracrine, as a consequence of its local synthesis and activity. It has marked reducing properties and has a vast range of cellular receptors both in the strict sense and in non-classical binding sites (Varga *et al.* 2001). The above mentioned facts is the reason why the complete mechanism of its action in hematopoiesis and in many other processes is not yet fully known.

Effect of MLT on differentiated blood and immunocompetent cells appears to be plausible, though not demonstrated. It remains mostly to be defined as regards to its possible role in the differentiating processes of the hematopoietic compartment such as hematopoietic and primary lymphoid organs.

OBJECTIVES

The aim of the current study was to assess the effects of MLT at pharmacological doses on limiting leukopenia and anaemia in young rats induced by CP during acute toxicity test at 12, 48 and 72 hours after administration.

CP is a prodrug which is activated through microsomal hydroxylation by cytochrome P450. Once hydrosilated in position 4, the molecule undergoes a non-enzymatic cleavage with the formation of acrolein and phosphoramidic mustard. The latter release orthophosphoric acid and ammonia leading to the formation of nitrogen mustard, a compound which shows an alkylating action towards the N7 position of guanine.

In the rat, unlike in man, at doses of over 50 mg/kg bw CP rapidly induces leukopenia and anaemia just 12–48 hours post-treatment (Paterson & Hanson, 1969; Wheeler *et al.* 2004). Specifically, Wistar and Lewis rats appear to be particularly susceptible to the alkylating action of this drug. Despite various studies have underlined the cytoprotective role of MLT against various genotoxic and alkylating agents (Caroleo *et al.* 1994; Melchiorri *et al.* 1998; Rapozzi *et al.* 1998; Vijayalaxmi *et al.* 1999; Koc *et al.* 2002; Lialiaris *et al.* 2008), and despite the fact that some studies have even shown a possible clinical use in reducing the toxic effects of

chemotherapy (Lissoni *et al.* 1995, a; Lissoni *et al.* 1995, b; Lissoni *et al.* 1996), other clinical studies have put forward the absence of efficacy of MLT in reducing leukopenia and anaemia in patients subjected to anticancer chemotherapy, suggesting however its protective role in reducing oxidative stress (Ghielmini *et al.* 1999; Sarma *et al.* 2004).

On the basis of the above considerations, we wondered if, in the short term, MLT could possibly have a protective role against leukopenia and in general against hematic cytopenia induced by treatment with CP, bearing in mind that, longer term, the onset of severe toxic consequences may decrease MLT's trophic effect on the BM. The protective role of MLT was earlier suggested by studies performed on animals irradiated and contemporaneously treated with MLT (Vijayalaxmi *et al.* 1999; Koc *et al.* 2002).

In our study MLT was administered at a dose of 10 mg/kg bw to assess its effect at 12 and 48 hours, while subsequent evaluation at 72 hour post-treatment was carried out following further administrations of MLT at 24 and 48 hours after the first administration. As some of the properties of MLT can be attributed to its reducing action, a parallel evaluation at 72 hours was carried out to determine the action of L-ascorbic acid (vitamin C) and of α-tocopheryl acetate (vitamin E).

The qualitative and quantitative composition of the blood was studied via a hemocytometric analysis and an examination with the classic Romanowski staining.

METHODS

In this study 50–65 days old Wistar rats, weighting 220 ± 16 g were utilized. These rats came from breeders specialised in supplying animals for laboratory research. Once in our laboratory, the rats were reared and treated with the utmost respect for their physiology as specified in the relative guidelines: they were fed *ad libitum* with a standard balanced diet and kept in an environment with alternation of light/dark of 12 hours at a temperature of 20–22 °C.

MLT (Sigma Aldrich, purity 98%) was solubilised in dimethylsulphoxide (DMSO) (Sigma Aldrich, purity 98%) and brought up to volume with sterile physiological solution (NaCl 0,9%). Solutions were prepared containing 200 mg/100 ml of MLT in saline solution with 2% w/V DMSO.

CP (Baxter, purity 98%) was dissolved directly in sterile physiological solution. Solutions were prepared containing 500 mg/100 ml of CP in saline solution. These were administered immediately after preparation and care was taken to maintain them at a temperature below 25°C.

L-ascorbic acid (vitamin C) (Roche, purity 98%) was solubilised in sterile physiological solution. Solutions were prepared containing 200 mg/100 ml of vitamin C in saline solution with 2% w/V DMSO.

(±) α-tocopheryl acetate (vitamin E) (Roche, purity 98%) was used with no further preparatory modifications.

MLT, CP and vitamin C were administered subcutaneously in the dorsal panniculus adiposus. Vitamin E was administered orally by gavage.

To assess the effects of MLT on BM depression induced by CP, four groups of randomised rats were used. Two groups of animals received MLT at a dose of 10 mg/kg bw and CP at a dose of 75 mg/kg bw. An hour elapsed between the two administrations. The remaining two groups were administered a sterile physiological solution of 2% w/V DMSO and CP at a dose of 75 mg/kg bw. Similarly to the previous, an hour elapsed between the two administrations.

No animals showed evident signs of idiosyncrasy connected with the various administrations.

After 12 hours samples were taken from two groups of rats, the first treated with CP only and the second with both MLT and CP: the rats were anaesthetised with diethyl ether and subsequently 2–3 ml of blood were taken from the left ventricle of each rat using microperfusion needles. Blood was collected into sterile test tubes containing EDTA and temporarily maintained at +4°C. Blood samples were analysed within 6 hours from collection at the Bioanalisi Laboratory in Modena.

A slide for each sample was also prepared according to the classic May-Grunewald-Giemsa staining method: this enabled both direct cytological evaluation of the samples under examination and a double-check of the figures given by the device. Blood slides were subsequently analysed in our laboratory using a Zeiss optic photomicroscope.

The same sampling and laboratory analysis methods were used for the remaining two groups of rats in relation to the 48 hour assessment.

Following this session of study a new work was planned to evaluate the effects of repeated administrations of MLT on BM depression again induced by CP. The effect of antioxidant vitamins on BM depression induced by CP was also investigated.

Three groups of randomised rats were used. The first group of animals received MLT at a dose of 10 mg/kg bw and CP at a dose of 75 mg/kg bw with an hour elapsing between the two administrations. The second group received vitamin C at a dose of 7.6 mg/kg bw and CP at a dose of 75 mg/kg bw. Similarly, an hour elapsed between the two administrations. This group of rats received, in addition, vitamin E at a dose of 30 mg/kg bw. The third group received a sterile physiological solution of 2% w/V DMSO and CP at a dose of 75 mg/kg bw. In this case too, an hour elapsed between the two administrations.

After 24 and 48 hours had elapsed since this first treatment, administrations of MLT at a dose of 10 mg/kg bw were repeated for the first group of animals, vitamin C at a dose of 7.6 mg/kg bw and vitamin E at a dose of 30 mg/kg bw for the second group of rats, and a

sterile physiological solution of 2% w/V DMSO for the third group.

No animals showed evident signs of idiosyncrasy connected with the various administrations.

Seventy-two hours after first administration the test samples were taken from the three groups of rats using the same procedures as described previously.

Statistical analysis of data was carried out using a two-tailed Student t-test for unpaired data. Any variations with $p<0.05$ were considered significant.

RESULTS

Results after 12 hours

At 12 hours a significant difference between the two groups can be clearly seen in the number of leukocytes: the group treated with both MLT and CP shows a 47% increase in the number of white blood cells compared to that treated with CP alone (Table 1). The Student distribution shows a level of significance with $p<0.05$.

Results after 48 hours

At 48 hours the difference in the rate of circulating leukocytes appears much less marked than at 12 hours, despite two rats presenting a leukocyte value of over 5000/mm^3 in the group treated with MLT. A significant increase of 16% is instead detected in the number of red blood cells in the group of rats treated with MLT compared to that treated with CP only (Table 2 and 3). The Student distribution indicates a level of significance with $p<0.001$ and it is also interesting to note the concurring variations in hemoglobin rate, which in the group treated with MLT is 13% higher ($p<0.001$). In the same way the group of rats treated with both MLT and CP shows an increase of 15% in hematocrit ($p<0.001$) compared to the group treated with CP only.

Results after 72 hours

At 72 hours the hemocytometric analysis does not show statistically significant variations, although, similarly to the previous tests, a greater value of red blood cells corresponds to the MLT treated group, though this is not as great as the corresponding value at 48 hour. In addition, the number of circulating lymphocytes is slightly higher, both in the group treated with MLT and in the group treated with vitamins C and E, respectively +8.1% and +6.5% ($p<0.05$). With regard to the remaining values, the three groups show fairly homogenous results.

DISCUSSION

The data outlined above lead to a number of interesting observations. First of all, a single administration of MLT shows itself to be considerably active in delaying the effects of CP, completely annulling leukopenia in the initial hours after treatment, while with the passage of time its effects on leukopenia diminish. It also seems

Tab. 1. Hemocytometric analysis after 12 hours.

	CP	CP + MLT
WBC (/mm³)	5752.7 ± 577	8460.0 ± 1170
Lymphocytes (%)	80.6 ± 1.3	80.4 ± 1.2
RBC (10⁶/mm³)	6.876 ± 0.194	7.063 ± 0.197
HGB (g/dl)	13.15 ± 0.3	12.20 ± 1.3
HCT (%)	38.63 ± 0.9	39.23 ± 0.9
PLT (10³/mm³)	976.2 ± 42.6	957.8 ± 86.2

Tab. 2. Hemocytometric analysis after 48 hours.

	CP	CP + MLT
WBC (/mm³)	3207.7 ± 262.5	3246.7 ± 338
Lymphocytes (%)	74.7 ± 2.0	73.1 ± 2.4
RBC (10⁶/mm³)	6.005 ± 0.181	6.981 ± 0.160
HGB (g/dl)	11.67 ± 0.216	13.24 ± 0.3
HCT (%)	33.50 ± 0.6	38.62 ± 0.8
PLT (10³/mm³)	820.5 ± 56.9	752.9 ± 71.0

Tab. 3. Hemocytometric analysis after 72 hours.

	CP	CP + MLT	CP + Vitamins C and E
WBC (/mm³)	1154.5 ± 162	1118.2 ± 160	1109.1 ± 131
Lymphocytes (%)	80.03 ± 2.4	86.50 ± 1.2	85.22 ± 1.0
RBC (10⁶/mm³)	6.468 ± 0.137	6.731 ± 0.146	6.499 ± 0.150
HGB (g/dl)	12.32 ± 0.9	12.37 ± 0.2	12.28 ± 0.1
HCT (%)	35.61 ± 0.8	36.53 ± 0.7	35.63 ± 0.7
PLT (10³/mm³)	796.2 ± 30.5	783.9 ± 34.7	791.8 ± 32.4

interesting to note its marked action in augmenting the number of circulating red blood cells, an effect which reaches its maximum expression after single administration, 48 hours post-treatment and remains evident in all MLT treated rats albeit to different extents.

Experimental data from the trial with triple administration of MLT during 72 hours did not differ from single dose treatment, suggesting that repeated administration does not affect the results. Similarly, treatment with other reducing substances at pharmacological doses does not appear to significantly influence the action of CP, suggesting that an increase in reducing activity does not influence the effects of CP. However, it should be pointed out that at 72 hours both groups, treated with MLT and treated with antioxidant vitamins, significantly increased the level of circulating lymphocytes, a phenomenon which can be attributed to effects on leukocyte diapedesis. It is interesting to note how vitamin E reduces neutrophil diapedesis, by reducing the expression of P-selectin in the coronary endothelium of rats and ischemic patients subjected to angioplasty (Formigli *et al.* 1997).

These data together indicate that neither does MLT act on the alkylating activity of CP, nor does it reduce the antiproliferative potential of the drug. In the same way it does not influence the BM depression induced by the medication, a conclusion which is in agreement with the work of Ghielmini *et al.* (1999), nor does it seem that its protective action in relation to leukopenia are increased by repeated administrations.

The power of MLT to considerably diminish the toxic effects of CP on BM in the initial hours after administration seems to be, however, evident. The phenomenon observed might suggest that MLT plays a role in stimulating granulocytopoiesis and the activity of T lymphocytes, hypothesising that initially a stimulating effect on the formation of CFU-GM may prevail, the effect suggested by Haldar, Häussler and Gupta (1992, a; 1992, b) and other authors as well (Akbulut *et al.* 1999; Currier *et al.* 2000; Sutherland *et al.* 2002). A possible myelostimulating action of MLT might explain a similar modification in the early hours, in a way similar to what was observed in total BM irradiation studies (Koc *et al.* 2002). Since CP expresses its pharmacological effects by stimulating the apoptotic process, and considering that in many studies MLT has shown that it has a modulating action on apoptosis, probably by acting on the mitochondrial membrane, it may be hypothesised that at an initial stage, when the myelosuppressive effect of CP is observed, MLT delays the apoptotic process.

However, a direct effect on leukocyte diapedesis and on chemotaxis cannot be excluded, considering the fact that the plasmatic leukocyte pool is in equilibrium with pool in the tissue and the lymph circulation. Such possibility seems likely. On the other hand MLT seems to be able to modulate chemotaxis and endothelial permeability (Lotufo *et al.* 2006; Peña *et al.* 2007), as well as the expression and regulation of certain adhesion proteins (Kang *et al.* 2001). Similarly, an action of MLT on leukocyte turnover can not be excluded.

The important action of MLT in reducing CP-induced anaemia appears to be evident, the effect which was observed in all three experiments and which proves to reach its peak 48 hours after administration of the substances. Considering this fact, the plasma erythrocytic pool is in direct equilibrium with that of the BM, and to a much lesser extent with that of the splenic pool. These data appear to be significant in indicating
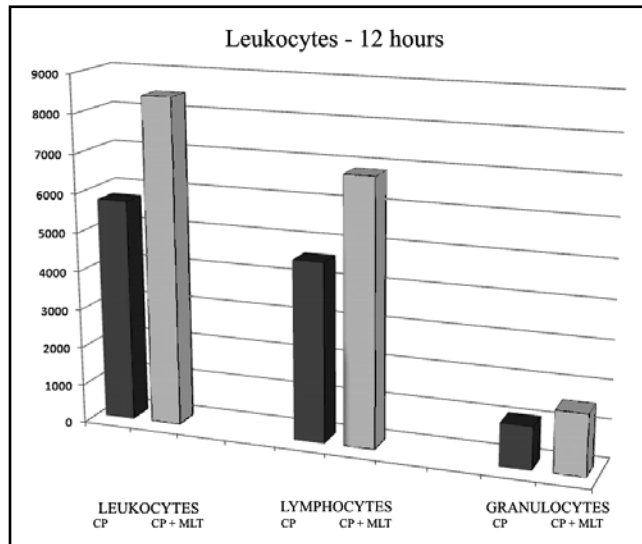
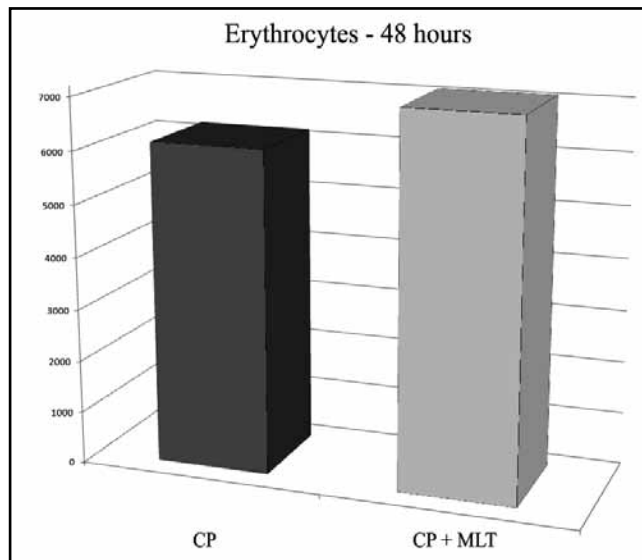**Fig. 1.** Leukocytes count 12 hours after administration of CP.

Fig. 2. Erythrocytes count 48 hours after administration of CP.
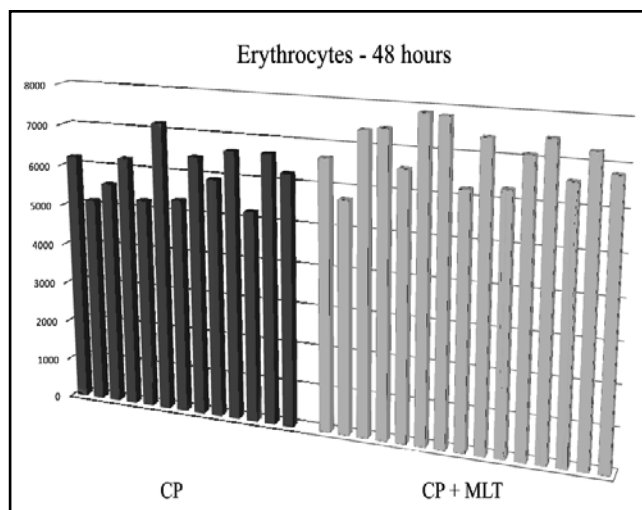

Fig. 3. Erythrocytes count in individuals 48 hours after administration of CP.

an action of MLT in stimulating erythropoiesis, or its direct effect on erythrocyte turnover.

An effective increase in the rate of circulating erythrocytes is also confirmed by the constant increase of the hematocrit and other hemocytometric parameters. The hypothesis of an action of MLT on erythropoiesis and/or on erythrocyte turnover does not seem impossible, especially when we consider the fact that nephrectomy in rats is associated not only with the evident reduction of erythropoietin but also with the massive fall in the levels of MLT, which in turn are normalised following administration of erythropoietin (Vaziri *et al.* 1996). In other clinical studies MLT has proved to be useful in reducing oxidative stress which follows the administration of erythropoietin and ferrous gluconate (Herrera *et al.* 2001; Hörl, 2002), while according to some evidence, chronic treatment with MLT induces an increase of circulating erythrocytes and hemoglobin in the elderly (Arushanian *et al.* 2006). Similarly, chronic administration of MLT in patients undergoing dialysis was shown to be useful in reducing anaemia and in preventing imbalance of iron metabolism (Labonia *et al.* 2005).

It is not superfluous to keep in mind that on circulating erythrocytes MLT was revealed to be efficacious in increasing the levels of reduced glutathione and decreasing those of glutathione reductase, highlighting a marked reduction of the erythrocyte membrane lipid peroxidation (Tesoriere *et al.* 1999; Kedziora-Kornatowska *et al.* 2007). Consequently, it seems that MLT may act to modulate the deformability of the erythrocyte membrane (Yerer *et al.* 2003). It should also be remembered that MLT is able to block erythrocyte carbonic anhydrase (Beydemir & Gülçin, 2004) and to stimulate the action of glucose-6-phosphate dehydrogenase (Ciftçi *et al.* 2001).

From the data acquired in this trial, and from the data published in the literature, it appears probable that MLT may affect erythropoietic process and/or erythrocyte turnover, as indeed suggested in previous studies conducted in our laboratory.

CONCLUSION

The present study is indicative in confirming the action of MLT on the hematopoietic system, suggesting its involvement not only in leukopoiesis, but also in erythropoiesis and/or in erythrocyte turnover. It is likely that the actions of MLT on the BM are multiple and heterogeneous, exerted through various antioxidant and receptor mechanisms as well as electrophysiological modulation. Lastly, it is clear that MLT significantly influences the effects of CP treatment, but only in the short post-administration period of the CP. Further studies will be needed to understand this phenomenon, which in any case seems indicative of the complex action of MLT, not just connected with its reducing potential, an activity which seems to be influenced by temporal interactions yet to be defined.

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