

MSC, A New Benzoquinone-containing Natural Product with Antimetastatic Effect

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An orally applicable fermentation product of wheat germ containing 0.04% substituted benzoquinone (MSC) has been invented by Hungarian chemists under the trade name of AVEMAR. Oral administration (3g/kg body weight) of MSC enhances blast transformation of splenic lymphocytes in mice. The same treatment shortens the survival time of skin grafts in a co-isogenic mouse skin transplantation model, pointing to the immune-reconstructive effect of MSC. A highly significant antimetastatic effect of MSC has been observed in three metastasis models (3LL-HH, B16, HCR-25). The antimetastatic effect of MSC - besides the immune-reconstitution - may also be due to its cell adhesion inhibitory, cell proliferation inhibitory, apoptosis enhancing, and antioxidant characteristics, also observed in our in vitro experiments. It is even more noteworthy that combined treatment with MSC and one of the following antineoplastic agents (5-FU and DTIC) - both in wide use in every day clinical practice - exhibited a significantly enhanced antimetastatic effect in appropriate metastasis models (established from C38 mouse colon carcinoma and B16 mouse melanoma respectively) as compared to the effect elicited by any component of these therapeutic compositions (MSC + 5-FU and MSC + DTIC) administered alone. The results show that the fermented wheat germ extract (MSC) has more than an additive effect and synergistically enhanced the metastasis inhibitory effect of both antineoplastic agents studied till now. It is also worthy of mention that the synchronous treatment with MSC profoundly decreased the toxic side effects of the applied antineoplastic agents (decreased weight loss etc). Based on the biological effects of MSC - shown to be non-toxic by subacute toxicology studies - this product may be used as an adjuvant in the therapy of malignant neoplasia and other diseases caused by or following immune-deficiency.

Key words: Fermented wheat germ extract, substituted benzoquinone, MSC, AVEMAR, metastasis-inhibition, immune-reconstruction, combination chemotherapy

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MATERIALS AND METHODS

Animals

In all experiments inbred mice from our Institute were used. The animals were 8-10 weeks old and weighed 20 - 22 g. They were kept in plastic cages (5 animals per cage) and were fed with rodent pellets (Charles River Hungary) and tap water *ad libitum*. The room temperature was 20-22°C, relative humidity $50 \pm 5\%$.

Tumor Models

The following transplantable tumor lines, grown on mice or rats, were used in the experiments: a highly metastatic variant of Lewis lung carcinoma (3LL-HH), B16 mouse melanoma, C38 mouse colorectal tumor and HCR-25, a human colon carcinoma xenograft.

The 3LL-HH, B16 and C38 mouse tumors were maintained on C57Bl/6 inbred mice.

The HCR-25 xenografts were inoculated into thymectomized, whole body irradiated and bone marrow reconstituted immune-deprived CBA/CA mice.

The highly metastatic variant of Lewis lung carcinoma (3LL-HH) was maintained in C57Bl/6 mice by serial intrasplenic transplantation of tumor cells obtained from liver metastases (3×10^3 cells).

The B16 melanoma line was maintained in C57Bl/6 mice by serial intramuscular transplantation (5×10^5 cells, hind leg muscle).

In case of the Lewis lung carcinoma (3LL-HH), single-cell suspensions were prepared from the liver metastases, while in case of the B16 melanoma the single-cell suspensions were prepared from the primary (muscle) tumor. The technique of preparing cell suspension was otherwise similar in case of both tumors. Tumor tissues were cut by crossed scalpels and filtered through fourfold gauze. After centrifugation (800 rpm) and washing (Medium 199) the viability of the cells was determined with trypan blue solution.

In the experiments with 3LL-HH, 3×10^2 or 3×10^3 cells were injected into the spleen of adult mice. Animals were sacrificed after 14 days of tumor inoculation. Primary tumor weight was determined by subtracting the average weight of spleens of ten

normal healthy mice of similar age from the weight of the tumor-bearing spleens. The number and diameter of liver metastases were determined by examining liver surfaces and counting the tumor colonies visible under stereomicroscope.

In the case of B16 melanoma, single-cell suspensions (prepared as described above) were obtained from 14-day-old primary tumors. 5×10^5 viable tumor cells were injected into the hind leg muscle of the animals. Primary tumor weights and number of lung metastases were determined after 21 days of tumor inoculation.

In the appropriate experiments, the primary tumor was removed by amputation of the tumor-bearing leg 10 days after tumor inoculation.

The HCR25 human colon carcinoma xenograft was established from a moderately differentiated primary human colon carcinoma in our Institute. The tumor was maintained in immunosuppressed CBA/CA mice by serial subcutaneous transplantation of small tumor fragments.

The C38 mouse colorectal carcinoma was maintained in C57Bl/6 mice by subcutaneous transplantation. The technique of preparing single cell suspensions in the case of HCR25 and C38 tumors was similar, as described below.

Single-cell suspensions were prepared from subcutaneously growing tumors. The tumor tissue was cut into small ($0.5 \times 0.5 \times 0.5$ mm) pieces in Medium 199, followed by enzymatic digestion at 36°C for 30 minutes under mild stirring in 10 ml Medium 199 containing a mixture of the following enzymes: collagenase I (Sigma-Aldrich) 0.0071 g, DNase (Sigma-Aldrich) 0.005 g and hyaluronidase (Sigma-Aldrich) 0.00044 g. Following digestion, the number of viable cells were determined and adjusted to 2×10^7 ml in case of the HCR25 tumor and set to 1.8×10^7 ml in case of the C38 tumor.

In our experiments the 3LL-HH, C38 and HCR25 tumor lines were used for establishing spleen-liver metastasis model, therefore under narcosis (Nembuthal, 70 mg/kg) the tumor cells in required number depending on the tumor type were implanted into the spleen as described below. A cross incision was made on the abdominal wall of the mice at the height of the spleen. The needle of the syringe containing the tumor suspension was inserted into the spleen parallel with the axis, and 0.1 ml of cell suspension containing the required number of tumor

cells was injected. The required number of tumor cells were the following: 3×10^2 or 3×10^3 cells in the case of 3LL-HH, 10^6 cells in the case of HCR25 and 8.8×10^4 cells in the case of HCR25 tumor lines. Bleeding at the place of insertion was stopped with a Gelaspon sponge. The abdomen was closed with Michael hooks, the lips of the wound were turned outside.

The B16 melanoma served as muscle-lung metastasis model by injecting the tumor cells in the muscle of the left hind leg as described above.

Treatment

MSC treatment was started 24 hours after tumor implantation. MSC was dissolved in water and administered by means of a gastric tube. The daily dose was 3 g/kg body weight *per os* administered in 0.1 ml of water. Control animals received tap water daily (0.1 ml), also *via* gastric tube.

The experiments were completed 14 (3LL-HH), 21 (B16), 20 (C38) and 51 (HCR-25) days after tumor inoculation, by means of exsanguination during anaesthesia.

RESULTS

The results of the experiments in which only MSC treatment alone was applied are summarized in Figures 2, 3 and 4.

MSC treatment resulted in a significant 71% decrease in the number of liver metastases of the 3LL-HH tumor inoculated into the spleen (Figure 2).

In case of the HCR-25 human colon carcinoma, the 50 days of MSC treatment decreased the amount of liver metastases, in addition to reducing the weight of the tumorous spleen (Figure 3). The number of metastases as compared to the control group of both the splenectomized and non-splenectomized MSC-treated animals was around 50%.

In case of the B16 melanoma inoculated into the muscle, there was no change in respect to mass of the tumor growing in the muscle, however, a significant decrease of 85% was observed in the number of metastases as compared to the control group (Figure 4).

Combined Use of AVEMAR and Cytostatics

In these experiments the following tumor strains were used: B16 mouse melanoma and C38 mouse colorectal tumor. The aim of these experiments was to find out how the daily treatment with MSC (3 g/kg body weight) would influence the tumor growth and metastasis inhibiting effect of treatment with some of the well known antineoplastic agents [5-Fluorouracil (5-FU) and Dacarbazine (DTIC)] widely used in clinical oncology in the frame of various treatment-protocols.

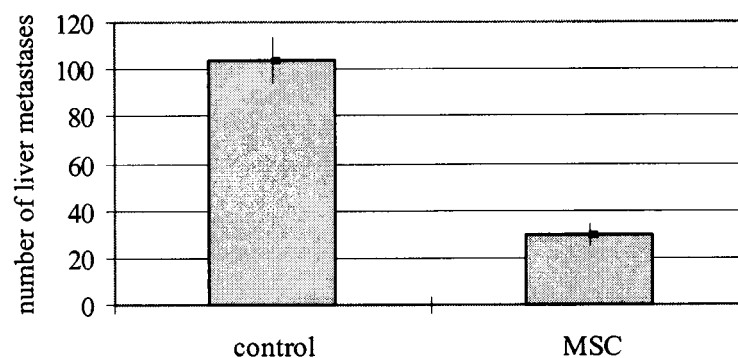


Figure 2. Effect of MSC on liver metastasis number of 3LL-HH carcinoma (mean±SEM).

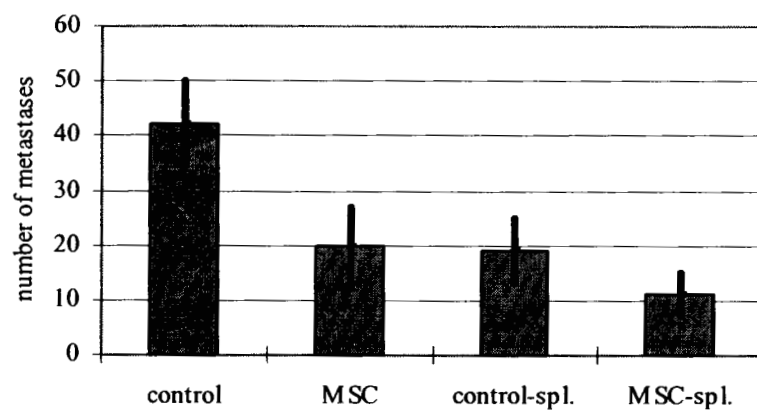


Figure 3. Effect of MSC on liver metastases of HCR25 human colorectal carcinoma (mean±SEM).

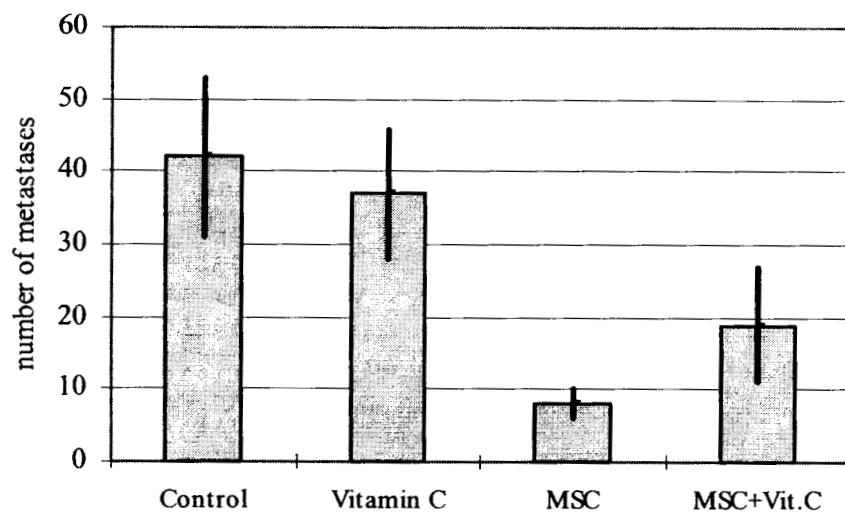


Figure 4. Effect of MSC on lung metastasis formation of the B16 melanoma (mean±SEM).

The B16 melanoma was used as muscle-lung metastasis model, while the C38 mouse colorectal carcinoma cell line was applied for serving as spleen-liver metastasis model.

Mice bearing the C38 colorectal carcinoma implanted into the spleen were treated with 5-FU

administered *i.p.* 3 times a week in a dosage of 1 mg/kg, while the mice inoculated with the B16 melanoma received DTIC treatment daily (60 mg/kg *i.p.*) Synchronously, the animals treated with antineoplastic agents also received MSC daily (3 g/kg). The experiments were terminated by bleeding out the

mice under narcosis on days 20 and 21 following tumor inoculation in the case of C38 tumor and B16 melanoma bearing animals, respectively. In these experiments the body mass of the animals was checked with particular care every three days. The size of the B16 melanoma growing in the muscle of hind leg was also measured every three days with a caliper. At the time of evaluation the animals were narcotized with diethylether and bled out. The mass (weight) of the primary tumor and of the organs with metastases was registered with an accuracy of 0.1 g. The number of liver and lung metastases was counted and their diameters accurately measured

with a scale built into a stereo microscope. The results are shown in Figures 5 and 6.

Figure 5 shows that in the case of combined (MSC + DTIC) treatment the number of lung metastases of B16 melanoma practically decreased to zero, and this effect is significant. The results show that in therapeutic composition, the metastasis inhibitory fermented plant extract (AVEMAR, MSC) - having metastasis inhibitory effect also alone - exerted a more than additive effect, that is, it synergically enhanced the metastasis inhibitory effect of DTIC used in clinical practice to decrease metastasis in protocols for treatment of patients with metastasis.

Treatment	Number of lung metastases average±SD
Control group	20,0±6,0
MSC 3g/kg/day p.o.	4,0±2,1*
DTIC 60 mg/kg/day i.p.	7,0±4,3*
MSC+DTIC	0,1±0,1**

*<0,01

**p<0,001

Figure 5. Effect of the therapeutic composition (MSC+DTIC) on the number of lung metastases of B16 melanoma inoculated into the muscle of the hind leg.

Treatment	Spleen mass (g) ±SD (SEM)	Liver mass (g) ±SD (SEM)	Number of metastases ±SD (SEM)
Control group	0,84± 0,32 (0,11)	0,61± 0,35 (0,12)	185± 104 (35)
MSC 3g/kg/day p.o.	0,86± 0,25 (0,08)	1,14 ±0,19 (0,06)	41± 34 (11)*
5FU 1 mg/kg/2 days i.p.	0,26 ±0,13 (0,04)	1,09± 0,36 (0,12)	16±17 (6)**
5FU+MSC	0,12 ±1,02 (0,008)	1,04± 0,21 (0,07)	2± 4 (1)**

*p<0,003

**p<0,001

Figure 6. Effect of the therapeutic composition (MSC+5FU) on liver metastases of C38 colorectal carcinoma and on the mass of the parent tumor containing spleen.

Figure 6 shows that a 20 days treatment of C38 colorectal carcinoma with the therapeutic composition of MSC and 5-FU decreased the number of liver metastases synergically. This effect is significant. The mass of the diseased spleen also displayed a marked decrease as a consequence of the treatment.

Although the therapeutic effects at both of the combination experiments were considerable, the usual toxic side effects of cytostatics, e.g. decrease of body mass were not observed.

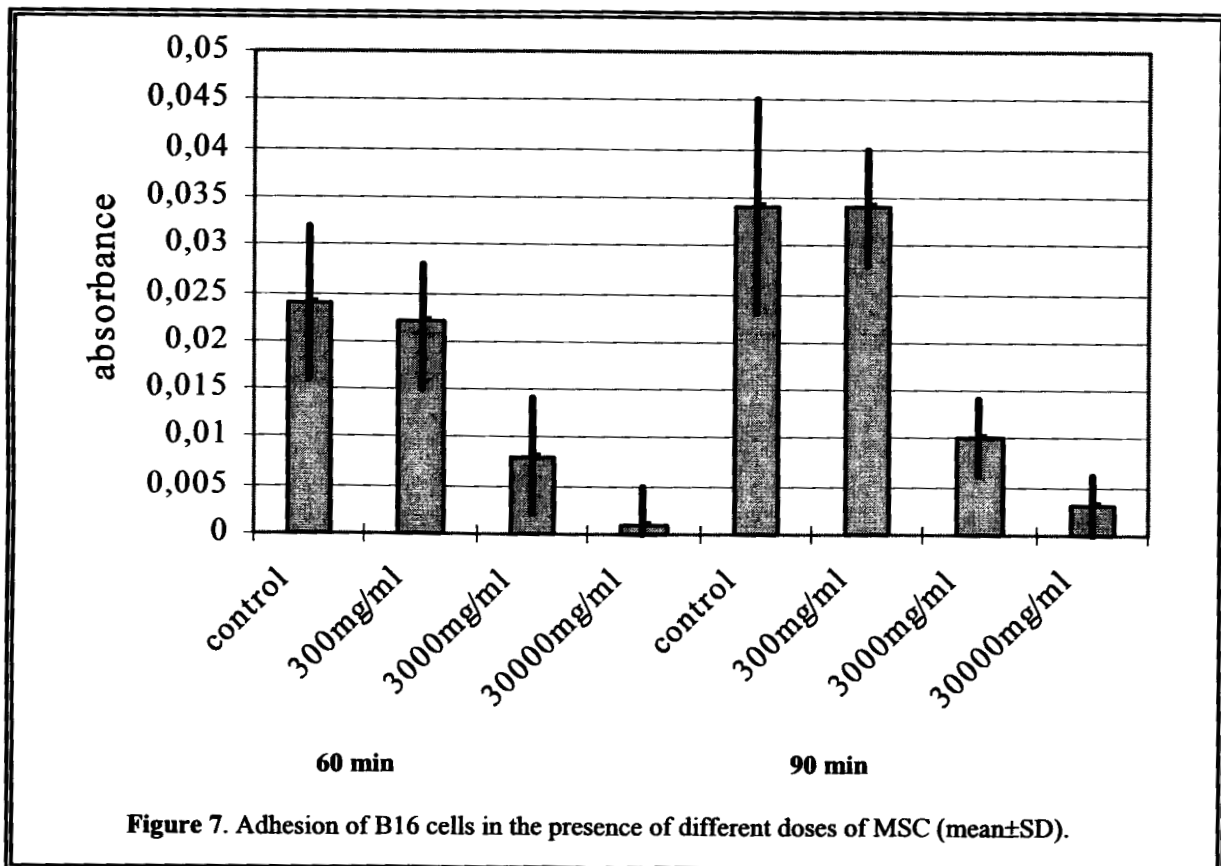
Studies on the actions of MSC

The above reviewed experiments refer to the fact that MSC brings forth a significant decrease in the amount of metastases in malignant tumors. Our studies aimed at clarifying the effect were therefore focused on certain important phases of metastatization. Metastasis formation is a multistep process in which the proliferative and apoptotic activity of the primary tumor cells, the adhesive ability of the tumor cells as well as the organism's defensive mechanism against the tumor cells all play role. In our *in vitro*

experiments we studied the effect of MSC on cell proliferation, apoptosis and adhesion.

Since a considerable part of our *in vivo* experiments were carried out on B16 melanoma cells in mice, this tumor was used for testing the preparation in our first series of experiments.

Cell adhesion was studied on a 96-hole plate, in the presence of the corresponding MSC dose in either serum-free or serum-containing medium, 10, 30, 60, 90 and 120 minutes after incubation in the usual tissue culture environment. Evaluation was based on the SRB test using colorimetric technique (the essence of the test is the sulphorhodamide B-staining of the culture's total protein content with absorbency read at 570 nm by means of spectrophotometry). Figure 7 shows two points in time which, however, demonstrate the effect of MSC adequately: the *in vivo* dose of 3000 µg/ml and naturally its tenfold concentration caused an equally dramatic decrease in tumor cell adhesion both in serum-free and serum-containing environments. This could not be said for the dose of 300 µg/ml.



In the **proliferation test** the cells were placed on a 96-hole plate 24 hours prior to treatment with MSC. Then 24, 48 and 72 hours after treatment the proliferation activity of the cells was determined with the help of the SRB test. Results of repeated experiments showed the tumor cells to float up from the monolayer in the 15000-900 $\mu\text{g/ml}$ domain as an effect of MSC. Furthermore, the tripane blue-staining elimination method proved that all the floating cells were destroyed (Fig. 8).

Our studies on human amelanotic melanoma (A2058) gave results similar to those of mouse melanotic melanoma (Fig. 9). Parallel with the SRB test, an MTT test representing cellular metabolic activity was also carried out. (The basis of this test is that by means of its dehydrogenase activity, the metabolically active cell transforms tetrazolium salt into chromatic formazane. The color reaction proportional to the activity was detected at 570 nm by spectrophotometry). It was established by the MTT test that even the dose of 300 μg decreased the functional activity of the tumor cells (Fig. 9). To detect why the cells that floated up had perished, flow cytometric (FACS) analysis was carried out to examine the apoptotic activity of the complete cell population (Fig. 10). The result was dose-dependent apoptosis of the cells in an unusually large proportion.

An interesting result necessitating further study was that after 24 hours no decrease was detectable by the SRB test in respect to either of the applied doses in healthy human fibroblasts - on the contrary, an increase was observable in several cases. At the same time, using the MTT test, doses of 3000 $\mu\text{g/ml}$ and larger exhibited significant decrease in metabolic activity while doses below 300 $\mu\text{g/ml}$ brought forth a considerable increase.

Experimental Studies on the Effect of MSC on the Immune Response

The **blastic transformation of T-lymphocytes** which plays a major role in the immune response showed a significant degree of increase as effect of the MSC treatment. This was verified by the following experiment. C57B1/6 mice were treated *per os* with 3 g/kg MSC using a gastric tube, for 6 weeks with doses given 5 times a week. After the treatment, lymphocytes taken from the spleen of the animals

by means of perfusion were placed into a cell culture for 1 $\mu\text{g/ml}$ of concanavalin A (ConA) treatment. 48 hours later the cells displaying DNA synthesis were marked with 0.4 μCi ^3H -thymidine. The degree of marking was measured with liquid scintillation counter (Beckman). As shown on Figure 11, ConA treatment significantly increased ^3H -TdR incorporation, that is blastic transformation as compared to the untreated control group.⁸

The reconstruction of the damaged immune response can best be modelled in co-isogenic **skin graft experiments** performed on partially immune-deficient thymectomized mice. The C57B1/10 and B₁₀LP mouse strains differ only in the H-3 locus, therefore skin transplanted from one strain to the other was not rejected within 7 days but approximately within 3 weeks. If the recipient was thymectomized, the average rejection period was about 50 days. All compounds promoting the maturation and differentiation of bone marrow lymphocytes similarly to thymic hormones shorten the time needed for skin graft rejection as shown by our next test on the effect of MSC treatment.⁹

The recipients were C57B1/10, the donors B₁₀LP mice. The recipient mice were thymectomized, then skin grafting was performed 7 weeks later. MSC treatment was started one week after the thymectomy, using a dose of 30 mg/kg by means of gastric tube given *per os* daily 5 times a week. Treatment was terminated 70 days after skin grafting. Observations of skin rejection were registered daily.

As shown on Figure 12, skin rejection in non-thymectomized mice took place in 21 (male) and 28.7 (female) days, and in 52.4 and 41.6 days, respectively, in thymectomized animals. MSC treatment considerably shortened the survival time of the skin grafts in the thymectomized and treated mice, substantially decreasing immune deficiency caused by thymectomy.⁸

Our *in vitro* and *in vivo* experiments with MSC refer to a very significant antimetastatic effect of this product observed in several animal experimental models. Most likely this effect correlates with the immune stimulatory effect manifested in both *in vivo* and *in vitro* tests, although the antiproliferative, apoptotic, adhesion-related, as well as free radical-forming effects may all contribute to the fewer number of metastases, too.

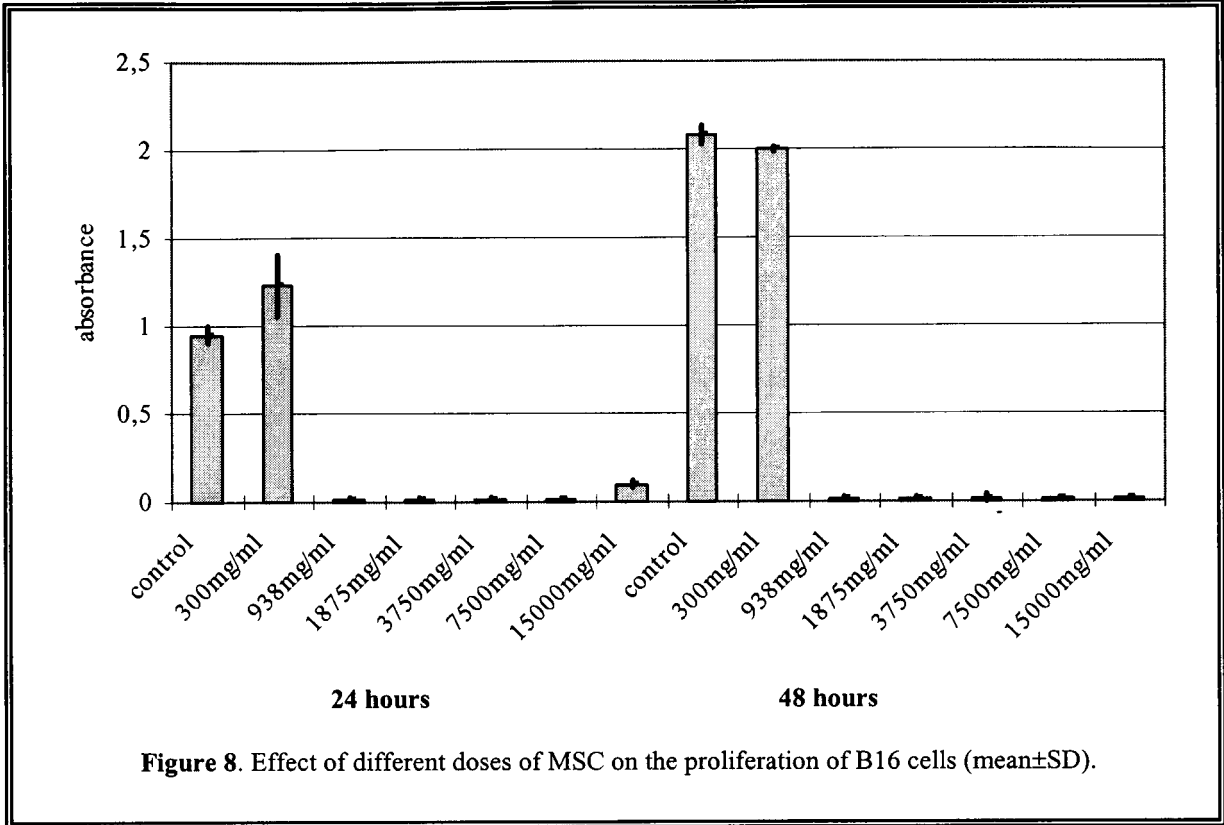


Figure 8. Effect of different doses of MSC on the proliferation of B16 cells (mean±SD).

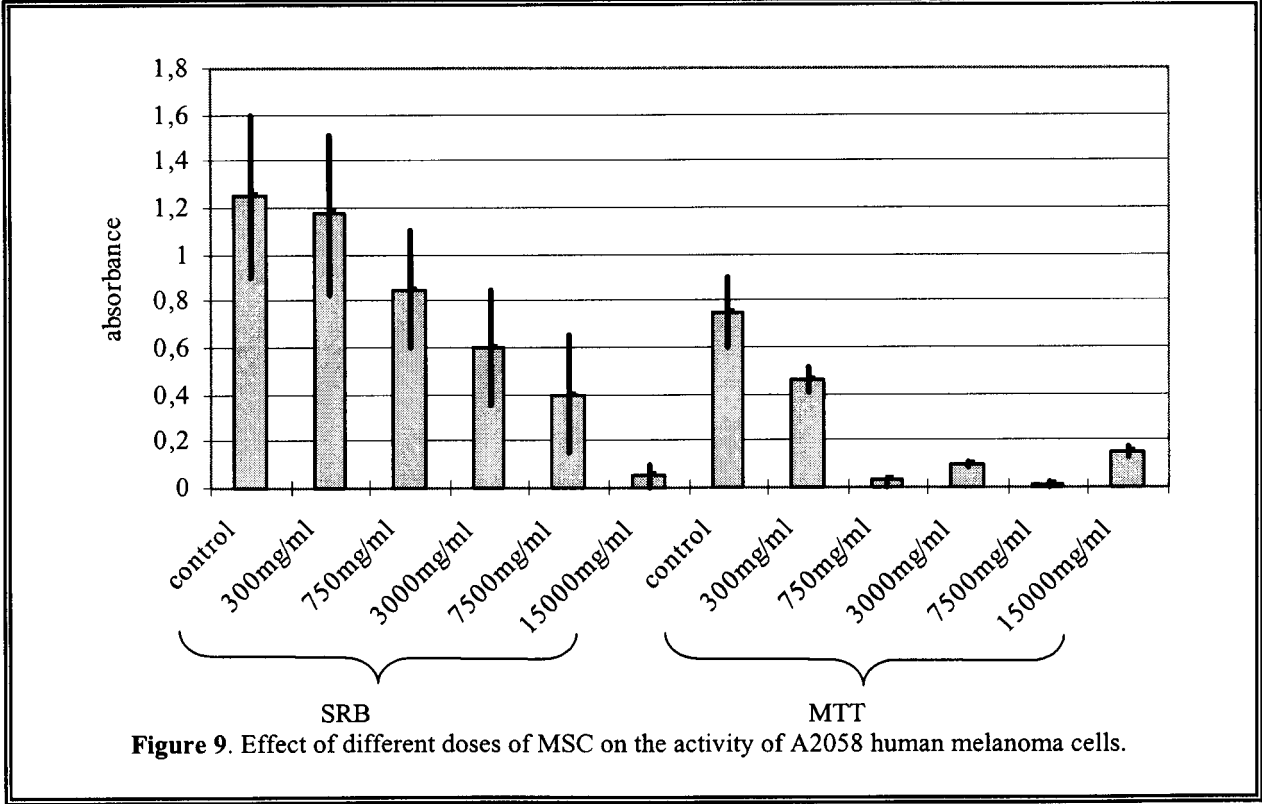
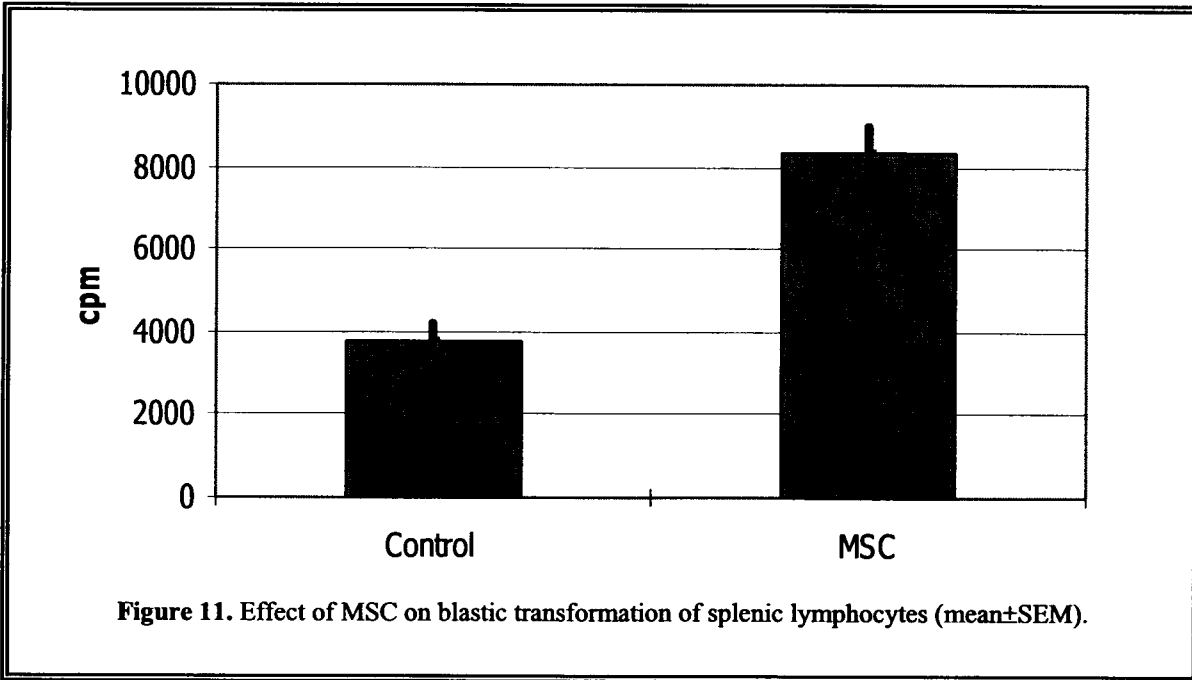
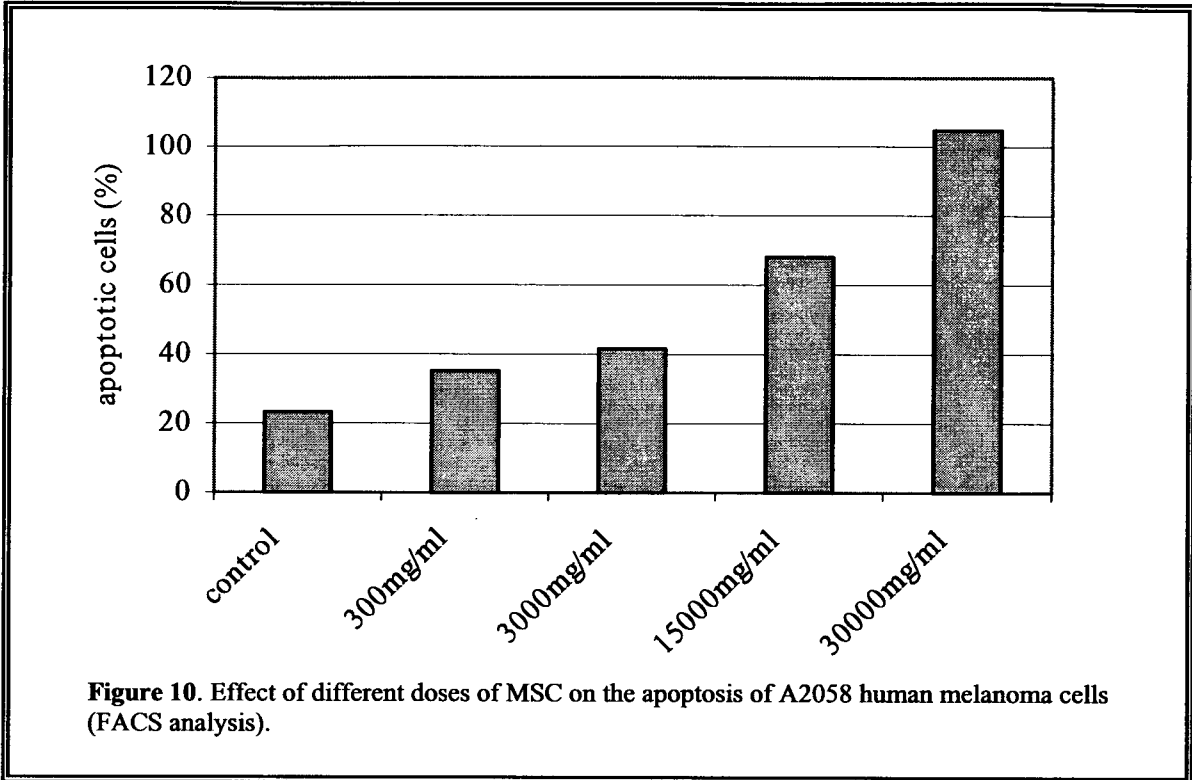
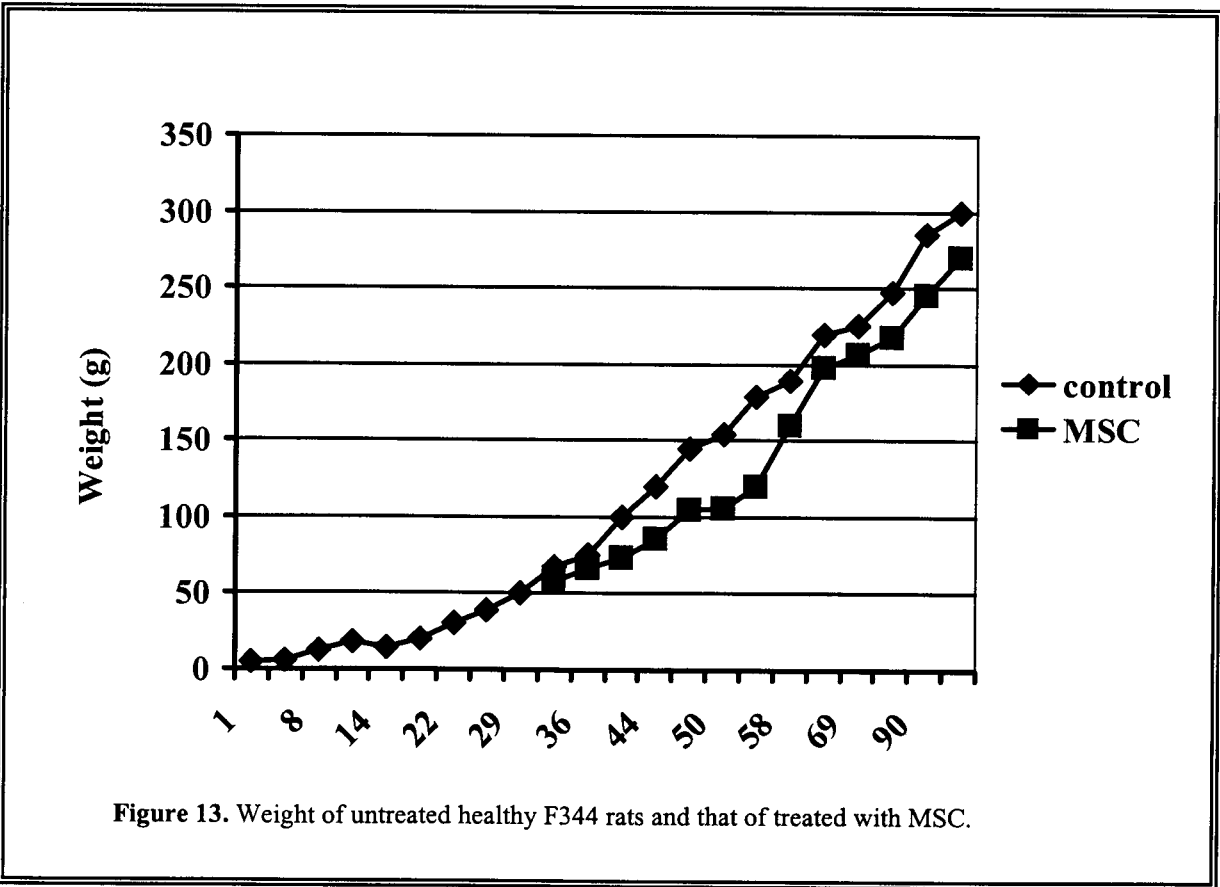
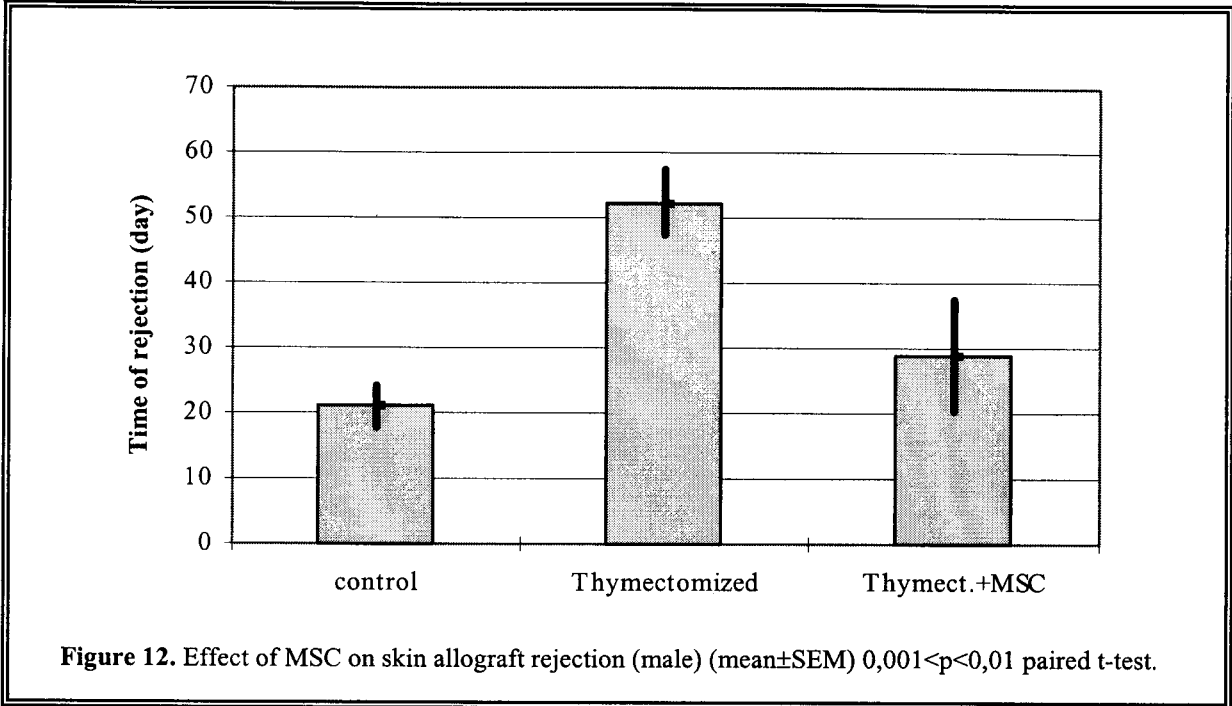
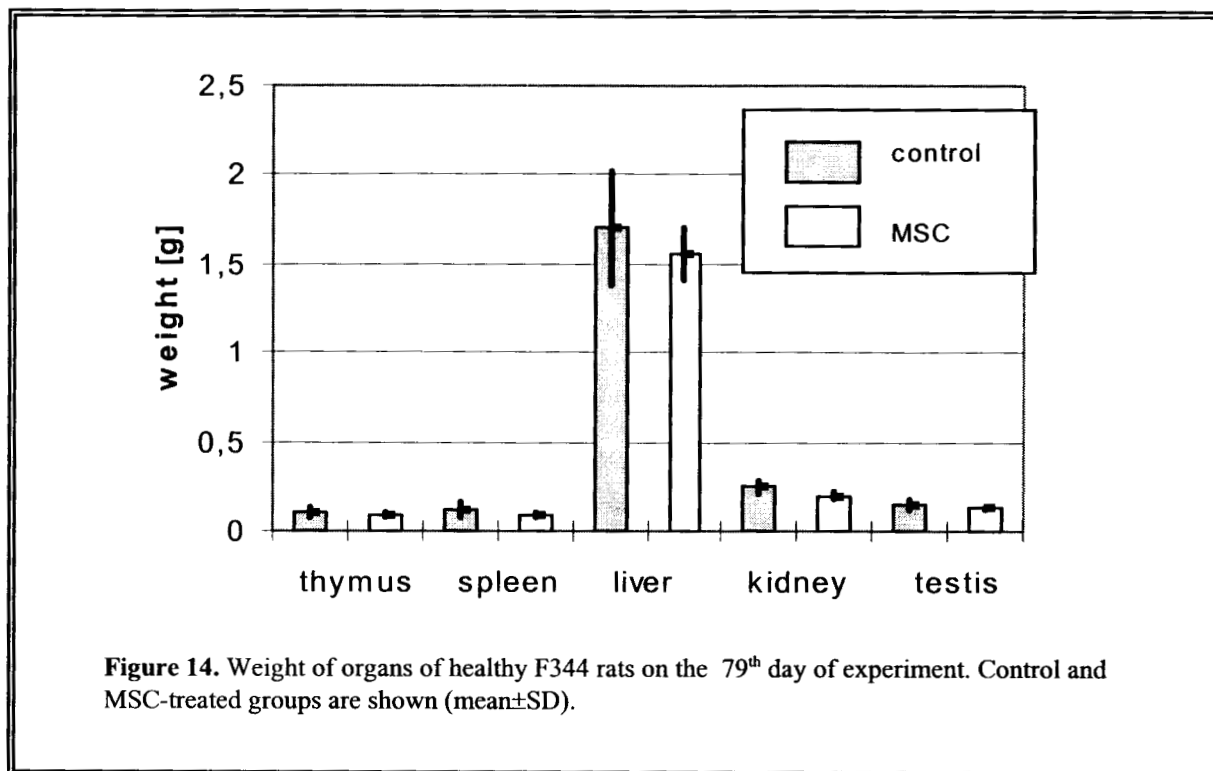


Figure 9. Effect of different doses of MSC on the activity of A2058 human melanoma cells.







The Free Radical Binding Effect of MSC

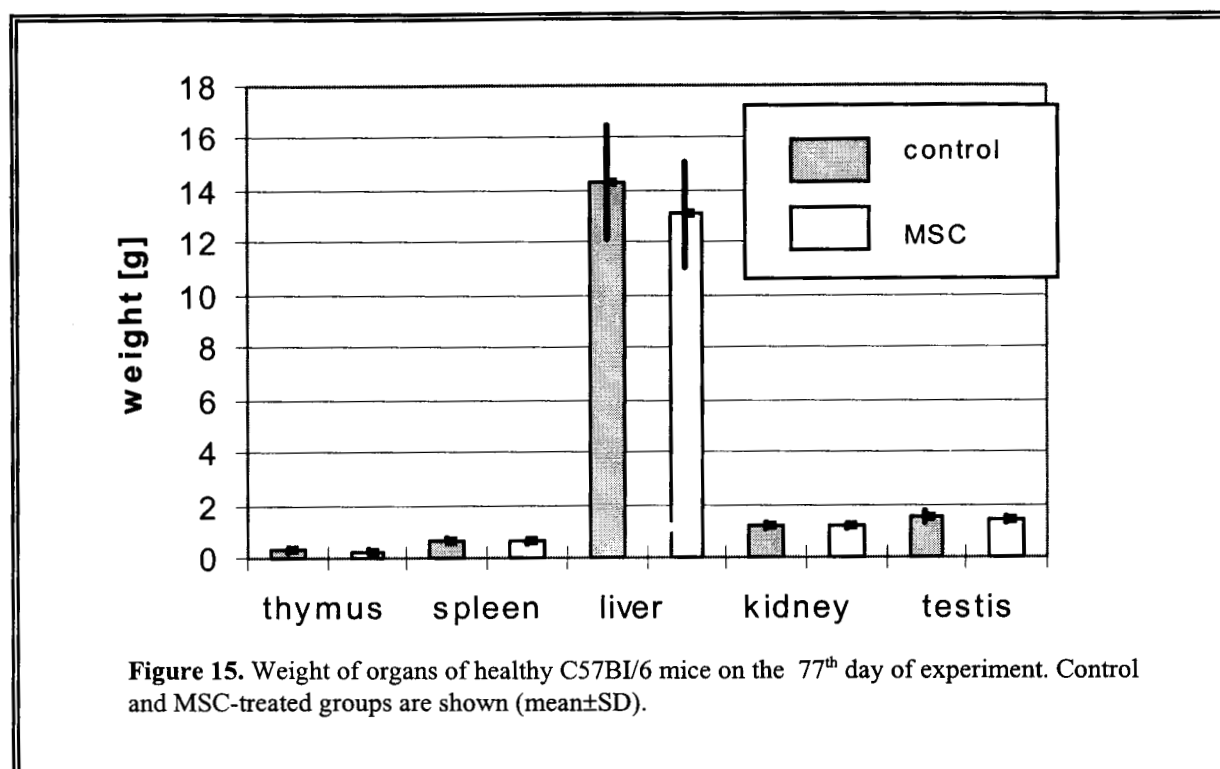
The biological and pathological significance of free radicals has been thoroughly studied during the past decade.¹ With respect to the reviewed effects of benzoquinones related to the formation of free radicals, it seemed necessary to study the free radical binding activity of MSC. Using electron spin resonance spin trapping¹⁰ method, both superoxide scavenging (SSA) and hydroxyl scavenging (OH-SA) were measured. There is considerable SSA related to MSC, with 1 mg of MSC having a scavenging capacity corresponding to that of 5.64 µg of superoxide dismutase (SOD). While MSC has no OH-SA activity, it interferes with the hydrogen-peroxide / Fe hydroxyl radical forming system, thus it could possibly possess a so-called non-chelator activity. Further study of the question is necessary.

Toxicological Studies

The 77 days' toxicological tests were performed in accordance with the Registry of Industrial Toxicology Animal data (RITA) recommendations,¹¹ using

F344 rats and C57B1/6 mice. The animals were treated daily, with a dose of 3 g/kg (in 0.6 g/ml dilution). During treatment the spontaneous death of the animals, as well as changes in body mass were registered. At the end of the experiment the mass of the following organs was recorded: heart, lung, thymus, spleen, liver, kidney and testis. Subsequently the 34 organs specified by the RITA were subjected to histopathological tests. No spontaneous deaths were observed and the body mass did not differ of the control group (Figs. 13, 14, 15). At the end of the experiment the organ masses did not show any deviation from the controls. The tests performed on the organs of the treated animals have not revealed any possibly harmful effects related to the treatment.

The product MSC has been put on the market as a non-toxic dietary supplement. Its immune-reconstructive effect recommends it for all conditions where the immune status has been injured. It is an adequate supplement to the pharmaceutical treatment of malignant tumors. Based on our *in vivo* and *in vitro* experimental data, the granulated product (AVEMAR) can be recommended in a daily dosage of 9 - 27 g.



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