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The in vivo Effects of Culture Medium. I. Radioprotective Effects of Vitamins, Amino Acids and Inorganic Salts of Culture Medium in Mice

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Summary

It has been shown that pretreatment of mice with a five-fold condensed culture medium before irradiation increased the number of multipotential haemopoietic stem cells in the spleen. The degree of cyto- and radioprotection is dependent on both the time of administration and the dose of the culture medium. The administration of culture medium in twofold doses in a volume of 1 ml intraperitoneally 18 h and 8 h before irradiation with a dose of 9 Gy protected 95 % of C57B1/6 mice. On the other hand, no therapeutic effect of different doses of the culture medium was found. Our observation suggests that the culture medium can mediate a radioprotective effect. The possible mechanisms participating in this effect are discussed.

Key words
Radioprotection – Vitamins – Amino acids – Survival – Colony-forming units (CFU)

Introduction

Exposure of mammals to ionizing radiation causes the development of complex, dose-dependent series of potentially fatal physiological and morphological changes know as the acute radiation syndrome. The systems that are least resistant to radiation damage are the lymphoid and haemopoietic tissues.

Recently, a large number of substances capable of protecting or having a positive effect on the recovery from radiation-induced damage in haemopoietic and lymphoid tissues has been tested. These chemical radioprotective substances differ as far as their chemical nature and the basic mechanism of their effects are concerned. However, the drawbacks such as undesirable toxic effects and limited time of protection are the main reason for testing the radioprotective properties of nontoxic biological substances. Naturally occurring compounds, such as vitamins, eicosanoids, selenium and similar substances belong to one of the basic groups of radioprotectants (Walker 1988). It has been suggested by the latest trends in radioprotection development (Weiss and Simic 1988) that these substances require new evaluation or reevaluation. Various "dietary precursors" belong to this group. Individual dietary supplements given alone or in different mixtures have been found

to be radioprotective in a number of experiments (for review see Perepelkin 1965). Some of them, e.g. pyridoxal (Artom *et al.* 1952, Morczek and Neumeister 1963, Schmidt *et al.* 1988), pantothenic acid (Artom 1954), amino acids and various protein hydrolysates (Jennings 1952, Sharpenak *et al.* 1959), cow milk (Jurášková 1971) or specially processed diets (Ershoff 1952, Pageau *et al.* 1975, Pageau and Bounous 1976, 1977) exhibited antiradiation effects and improved haemopoietic cell recovery after radiation.

The culture media comprise relatively balanced mixtures of different qualitative and quantitative contents of vitamins, amino acids and inorganic salts. Such mixtures providing optimum conditions for cells *in vitro*, contain components that can, *via* different cellular and molecular mechanisms, modify radiation damage. In our experiments, we have attempted to verify the possibility that a culture medium could mitigate the radiation effects *in vivo*.

We have demonstrated in this study that culture medium potently stimulates haemopoiesis in irradiated mice and that the animals receiving culture medium are protected against the acute radiation syndrome.

Material and Methods

Mice. Female C57B1/6 mice (Velaz, Prague) weighing about 20 g were used throughout this study. They were held for 14 days after delivery to allow for acclimatization to the new environment and for recovery from stress due to transport. Mice 10 to 12 weeks old, were housed in rodent cages, five to seven animals per cage. They were given Larsen diet (Velaz, Prague) and tap water *ad libitum*.

Table 1
Formula of culture medium used (in mg/l)

AMINO ACIDS		VITAMINS	
L-Alanine	8.9	Choline Chloride	2.0
L-Arginine . HCl	189.0	Folic Acid	2.0
L-Asparagine . H_2O	15.0	I-Inositol	4.0
L-Aspartic acid	13.3	Nicotinamid	2.0
L-Cystine	36.0	D-Pantothenic acid (calcium)	2.0
L-Glutamic acid	14.7	Pyridoxal . HCl	2.0
L-Glutamine	584.0	Riboflavin	0.2
Glycine	7.5	Thiamine . HCl	2.0
L-Histidine . HCl . H_2O	63.0	L-Isoleucine	78.7
L-Leucine	52.4		
L-Lysine . HCl	109.5		
L-Methionine	22.5	INORGANIC SALTS AND OTHERS	
L-Phenylalanine	48.0	$CaCl_2$	200.0
L-Proline	11.5	NaCl	6810.0
L-Serine	10.5	$MgSO_4 . 7 H_2O$	200.0
L-Threonine	72.0	KCl	400.0
L-Tryptophan	15.0	$NaH_2PO_4 . H_2O$	140.0
L-Tyrosine	54.0	Glucose	1000.0
L-Valine	69.0	Phenol Red	10.0

pH adjusted to 7.2 with 7.5 % sodium bicarbonate

Culture medium (CM). Minimum essential Eagle medium (MEM), non-essential amino acids (NEA), essential amino acids (EA), vitamins-MEM and L-glutamine were purchased from ÚSOL, Prague. Minimum essential Eagle medium was supplemented with NEA, EA, vitamins-MEM, L-glutamine and 10–15 % selected horse serum. The final composition of the medium is given in Tab. 1. Such a supplemented culture medium (in some experiments also horse serum) was lyophilized using Multi-Dry freeze dryer (FTS Systems, Inc., USA). Dry lyophilisate was dissolved immediately after lyophilisation in a 5-fold smaller volume of sterile deionized water compared with the original volume. Approximately 18 h and 8 h prior to irradiation, mice received an intraperitoneal (i.p.) injection of culture medium in a volume of 1 ml. Another animals were injected at the same time intervals and the same volume with amino acids alone, with vitamins-MEM alone and finally with "other components" of the culture medium alone (all dissolved in deionized water in the same concentrations as they were in a complete five-fold condensed culture medium). In the same way, mice received injections of lyophilized horse serum alone or of "natural" horse serum alone. However, because no significant differences were observed in the responses of mice receiving lyophilized horse serum or "natural" horse serum, data from all serum-treated mice were pooled. All culture media and its individual components were filtered through a 0.2 μm Minisart NML units (Sartorius, FRG). The injections were given at 9.00 p.m. and at 7.00 a.m. on the next day and irradiation was carried out at 3.00 p.m. In all experiments, control mice received i.p. saline of the same volume and the determinations were made concurrently with the treated groups.

Irradiation. Mice were placed in plexiglass containers and exposed to 9 Gy of total body gamma rays at a dose rate of 0.3 Gy/min. The Chisostat ^{60}Co source (Chirana, CSFR) was used for all irradiations.

Survival assays. Studies were initiated in November and completed in September. Irradiated mice were returned to the animal facility and treated routinely. Survival was monitored daily and was reported as the percentage of animals surviving 30 days after irradiation . Each treated group within each experiment consisted of 15–30 mice. Experiments were repeated 2–3 times to obtain at least 50 animals in each treated group. The percentage of mice surviving the radiation dose after 30 days after exposure was used to construct a probit plot survival curve for each treated group.

Haemopoietic stem cell assays. The effects of various inocula and time of injection prior to irradiation on the haemopoietic recovery were evaluated by the endogenous spleen colony-forming unit (E-CFU) assay (Till and McCulloch 1963). Briefly, mice received a dose of 9 Gy. They were killed on day 12 after irradiation by cervical dislocation, and their spleens were removed and fixed in Bouin's solution. The number of grossly visible colonies *per* spleen was determined and the mean and standard error were calculated. The treated groups within each experiment consisted of 5–10 mice each. Experiments were repeated 3 times to obtain at least 20 animals per group. The survival of haemopoietic stem cells within 365 days of postirradiation period was determined by exogenous spleen colony-forming unit (CFU-S) assay (Till and McCulloch 1961). The number of nucleated bone marrow cells was determined using a Bürker chamber after thorough rinsing of the femur with Hanks' solution and 10^5 cells were intravenously injected (*via* lateral tail veins) into lethally irradiated recipient mice. The recipient mice (n = 10 per group) received a 10 Gy dose of ^{60}Co radiation 24 h before the injection of test cells, and the numbers of CFU-S per spleen were determined 12 days later.

Statistical significance was evaluated by Student's t-test.

Results

Pretreatment of mice with 1 ml of culture medium (CM) before irradiation increased the number of endogenous haemopoietic stem cells (E-CFU). Fig. 1 shows that the time of CM injection was a critical factor for the expression of E-CFU. The culture medium produced the greatest increase in E-CFU if administered 30–60 min or 18–24 h before irradiation, with a maximum value obtained at 18 h prior to irradiation. Reinjection of the same dose of culture medium (administered at 18 h and 8 h prior to irradiation) resulted in a threefold

increase in the number of E-CFU (Fig. 1) as compared with the maximum value obtained after a single dose given 18 h before irradiation.

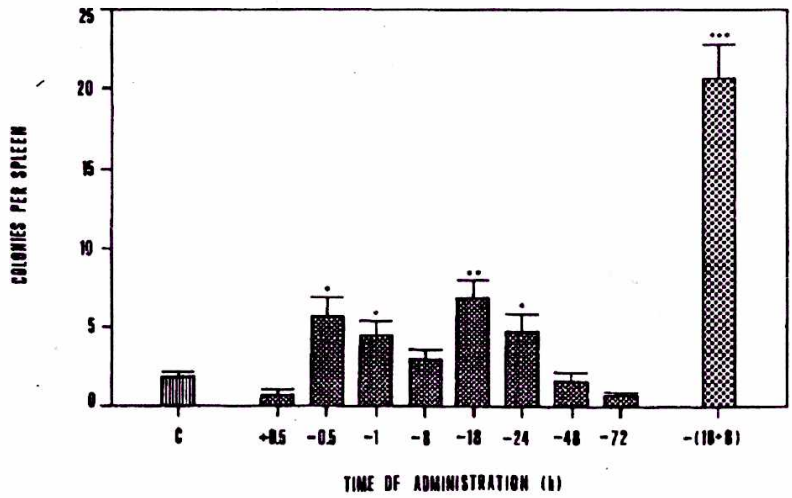

Fig. 1
Effect of time of culture medium administration on endogenous spleen colonies (E-CFU). Groups of mice received an i.p. injection of 1 ml CM as described in Materials and Methods. Control mice received i.p. saline in the same volume as the treated groups. Since no significant differences were observed in the responses of mice receiving saline at different time intervals, data from saline-treated mice were pooled. The results are means ± S.E.M. of three experiments. Treated groups represents 20-24 mice. Control data represent 62 mice. C – control, + – $P<0.05$, ++ – $P<0.001$, +++ – $P<0.0001$

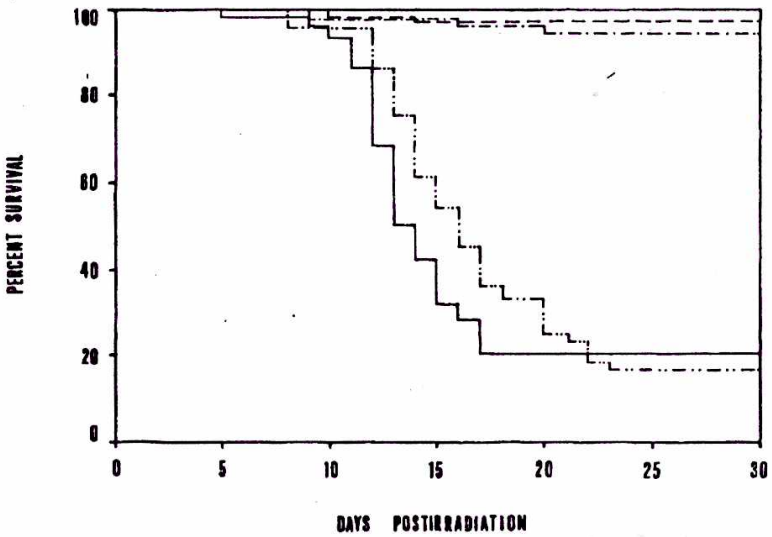

Fig. 2
Radioprotective effect of culture medium in irradiated (9 Gy) C57B1/6 mice. Mice received an i.p. injection of 1 ml of CM with horse serum (-.-.-, n = 72), serum-free CM (-----, n = 50), horse serum (-..-.., n = 84) or saline (___, n = 50) 18 h and 8 h before irradiation. The results are the means of three separate experiments.

A more detailed analysis of radioprotectant activities is illustrated in Fig. 2, showing 30-day survival profiles in each of the treated groups. The complete culture medium (with horse serum) protected C57B1/6 mice from lethal effects of ionizing radiation when it was administered intraperitoneally in double doses (1 ml 18 h and 8 h) before irradiation with 9 Gy (Fig. 2). When serum-free culture medium, "natural" horse serum alone or lyophilized horse serum alone (data from all serum-treated mice were pooled) were injected, more than 95 % of serum-free CM-pretreated mice survived as compared to only 16 % survival of horse serum-pretreated mice (Fig. 2). Administration of individual components of culture medium (amino acids alone, vitamins-MEM alone or "other compounds" alone) had no marked protective effect (data not shown). The degree of protection depended on both the time of pretreatment and the dose of CM injected. Administration of culture medium in a single dose 30 min or 8 h before irradiation resulted in a lower degree of protection (25 % survival as compared 95 % survival after double doses given at 18 h and 8 h before irradiation). Postirradiation CM injection (30 min after irradiation) appeared to be ineffective.

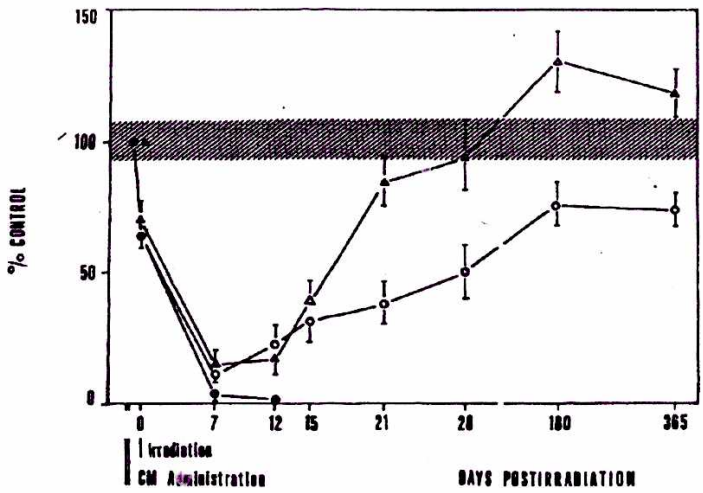

Fig. 3

Radiation survival curve for bone marrow CFU-S (open triangles) and bone marrow cellularity of mice injected with saline (closed circles) or CM (open circles). Mice were administered 1 ml CM 18 h and 8 h prior to their exposure to 9 Gy gamma radiation. Cells were pooled from both femurs of seven mice per group, and number of CFU-S per femur was determined. Values represent means ± S.E.M. of colonies from ten spleens per group.

The time interval that elapsed between irradiation and death suggests that death was primarily caused by bone marrow failure. This is also indicated by a considerable reduction in bone marrow cellularity (Fig. 3) found in saline-pretreated mice within days 7 to 12 after irradiation. Because of a small number of nucleated cells in the bone marrow of these animals, the exocolony spleen test was negative. In CM-pretreated mice, an initial depression was followed by continuous regeneration of bone marrow. It can be seen as a rapid increase in the CFU-S number, reaching control values as early as 21 days after irradiation. Similarly their marrow colony-forming activity was higher at later intervals than in control animals (Fig. 3).

Discussion

It follows from the present results that a lag period between its administration and irradiation of animals is necessary for a protective effect of culture medium. During this period a stimulation of the organism, namely of haemopoietic organs, occurs. In the radiation dose used by us, it is the recovery of haemopoietic cells to normal values which is considered to be the most important precondition of survival of irradiated mice. The culture medium increased the number of multipotential haemopoietic stem cells in the spleen. The intensity of stimulation is dependent on both the volume of culture medium injected and the time of injection. The graphic expression of this time-effect relationship is similar in shape to that found by Jurášková (1971) for the effect of cow milk. The increased postirradiation incidence of CFU-S and E-CFU and other manifestations of regeneration of this organ in CM-pretreated mice (Macková *et al.* 1991) might hint at a direct or indirect activation of stem cells during or at early intervals following irradiation. Different mechanisms can underlie this stimulation. One of them participating in the protective action, may be the more intensive proliferation (Kozubík and Pospíšil 1985) and entrance of stem cells into the more radioresistant phase of the cell cycle (Denekamp 1986) supported by the necessity of a time interval between the culture medium administration and irradiation (Boggs *et al.* 1972). When estimating the protective mechanisms it should also be noted that the mixture was given in an anisotonic solution because this could induce a physiological stress reaction with subsequent mobilization of defense mechanisms. Indeed, the administration of an anisotonic solution of inorganic salts (free vitamins and amino acids) to mice which were handled in the same way as CM-pretreated mice only exhibited a slightly increased resistance.

Do these radioprotective effects depend on humoral or cellular mechanisms, or both? Considering the broad scale of biological activities of each dietary factor it can be assumed that both mechanisms may overlap or complement each other. They are both essential for the recovery from haemopoietic injury due to ionizing radiation. Therefore, it is difficult to state explicitly the actual mechanism of this action. As yet we can only hypothesize about the possible routes leading to radioprotection by culture medium on the basis of the biological properties and effects of individual components of culture medium used.

An active form of vitamin B-6, pyridoxal-5'-phosphate, is required for more than 100 enzymes as an activating coenzyme, including nucleic acid synthesis. Hence, there exist numerous interactions between these and other compounds which establish a general status of the organism as well as a normal immune response (Chandra and Puri 1985, Sauberlich 1985, McCormick 1989). The administration of dietary supplements as employed by us may influence various metabolic processes and this situation may persist for a certain time after irradiation.

It is well known that some amino acids contribute to the enhancement of gluconeogenesis (Kaloyianni and Freedland 1990). Radiobiological experiments have shown that the enhancement of metabolites turnover as well as the improved utilization and mobilization of energy reserves activating lipogenic, glycogenosynthetic and gluconeogenic systems are inevitable preconditions of postirradiation recovery (Kozubík *et al.* 1985, Kozubík and Pospíšil 1985). These

authors have suggested that the increased metabolic rate and the accelerated recovery of haemopoietic functions after irradiation in metabolically activated animals are interrelated processes. Michaelson and Odland (1962) demonstrated that animal species with a higher intensity of metabolism also have a higher level of repair and more rapid recovery of haemopoietic tissues after irradiation, which correlates with the increased radiation resistance of these animals.

According to Artom (1954), the protective role of pantothenic acid is based on the maintenance of coenzyme A level in tissues. A crucial role of coenzyme A in metabolism and its role in the synthesis of porphyrins and heme is generally accepted. Some porphyrins, as was shown by Canti *et al.* (1989), are capable of modulating the haemolymphopoietic system in virgin and immunosuppressed animals.

Furthermore, some nicotinamide analogues play an active role in increasing the concentration of cAMP (Campbell *et al.* 1989), B6 vitamers in increasing the tissue magnesium level (Majumdar and Boylan 1989) and in recovering the chemically-induced lymphopenia (Gobin and Paine 1989). Adenosine and adenosine phosphates were found to be radioprotective in experimental animals (Grant *et al.* 1976, Tikhomirova *et al.* 1984, Pospíšil *et al.* 1988, Szeinfeld 1990). In addition, these substances have been suggested to be involved in red blood cell differentiation in the murine spleen (Winkert *et al.* 1971) and bone marrow (Bottomley *et al.* 1971). Pretreatment with culture medium, similarly as with a number of radioprotective agents, also induced erythropoiesis in irradiated mice besides splenic myelopoiesis, megakaryocytopoiesis and lymphopoiesis (Macková *et al.* 1991). However, the enhancement of erythropoietic recovery itself could not be responsible for increased survival, but this is probably representative of greater overall haemopoietic recovery (Ross and Peeke 1986). Wiemer *et al.* (1978) have shown that magnesium is a positive allosteric effector of adenylate cyclase that can raise cAMP levels. The results of Pospíšil *et al.* (1988) suggest that AMP and Mg^{2+} ions exhibit some synergism of the radioprotective action as revealed by the survival of lethally irradiated mice.

Nicotinamide is a precursor of cell NAD and its injection to laboratory animals was shown to have a radiosensitizing effect on tumors (Horsman *et al.* 1987). Based on their own experimental results, Riklis *et al.* (1988) have suggested that presence of nicotinamide during exposure to gamma radiation is aiding the repair synthesis and strand break rejoining by keeping the level of NAD optimal and enabling its utilization for the continued production of polyadenosinediphosphoribose. This is interesting and important factor in the biochemical events that may be linked to improved radioprotection (Riklis *et al.* 1988). From this point of view the results of Inoue *et al.* (1989) are relevant. These authors followed the effect of pyrazinamide, tryptophan or nicotinic acid and gamma-ray irradiation on NAD levels in various mice organs. These dietary factors are also capable of keeping the NAD at a higher level after irradiation as compared with that in control irradiated animals.

It follows from the present results, that the mutual interaction of the effects of individual components of culture medium in sequence is an essential condition for its radioprotective action. This suggestion is indirectly supported by the fact that isolated constituents of culture medium had no marked protective effect. In contrary, administration of amino acids alone lowered the survival of animals as

compared to that of irradiated control mice. On the other hand, it is still questionable to what extent this commercially supplied culture medium is optimal for the protective effect as far as the individual dietary factors are concerned. It is probable that a change in the proportion of its individual components will alter its protective effectiveness. A more detailed study is still required.

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