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Enhancement of liver size by stimulation of intact rat liver with exogenous hepatotrophic factors

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In mammals, liver size is related to animal body weight at the 2.5 to 3% proportion, a ratio mediated by the afflux of hepatotrophic factors. Formulas capable of modifying this ratio have been developed in previous studies on the rat, with enhancement of liver size brought about by intraperitoneal (portal) infusion of exogenous factors such as glucose, amino acids, insulin, glucagon, vitamins, electrolytes, and triiodothyronine. However, the efficacy of these formulations was accompanied by increased animal mortality (PARRA et al.[19,20]). The present study, which was carried out with small methodological modifications on a larger number of rats using daily intraperitoneal injections of a solution of exogenous hepatotrophic factors (40 ml/kg) for seven days, confirms the previous findings, with a 114.16 ± 7.90% enhancement of liver size beyond the expected value for the body weight of the animal. However, the problem of animal mortality was not fully resolved.

UNITERMS: Glucagon. Insulin. Liver regeneration. Thyroid hormone.

The liver has the ability to regulate its own size according to the trophic "influence" it receives, i.e., according to the afflux of the set of substances and hormones generically called hepatotrophic factors, many of them of splanchnic origin, such as insulin, glucagon, amino acids, vitamins, and certainly many more of still unknown origin. Thus, there is a specific liver size for each animal corresponding to 2.5 to 3% of body weight (2,7,18,24). This relation tends to remain constant through a process of cell multiplication known as regeneration which is activated whenever the organ loses part of its mass due to surgical (hepatectomy) or pathological (necrosis) reasons. The most intimate mechanisms underlying this control are not fully known. They involve modifications in the expression of several genes and the appearance of messenger RNA and its respective protein molecules which represent several other factors having either a stimulatory action such as hepatocyte growth factor(HGF) and transforming growth factor α (α-GTF) or an inhibitory action on this process such as transforming growth factor β (β-TGF) (14,21). In practical terms, there is general acceptance of the existence of an equilibrium between liver size and supply of splanchnic hepatotrophic factors.

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Previous studies by our group have demonstrated that this equilibrium can be broken by exogenous supplementation with some known hepatotrophic factors, leading to an increase in intact (non-hepatectomized) liver size beyond the biologically predetermined size(19,20). The successive formulations developed in these studies have shown an increasing ability to stimulate a regenerative enhancement of liver size, although at the expense of also increasing mortality, thus preventing statistical validation of the data obtained due to the small number of surviving animals. The objective of the present study was to confirm the previous findings using small methodological modifications and a larger number of animals.

MATERIAL AND METHODS

Female Wistar rats weighing approximately 200g were divided into two groups and housed in cages with food and water "ad libitum". After a 24-hour fast, the animals were identified individually and weighed and then injected daily intraperitoneally with 40 ml/kg of the following solutions:

Group A (control, N = 10) - Saline solution containing carboxymethylcellulose (CMC) at 0.25% concentration.

Group B (N = 20) - a standard solution for parenteral nutrition of the following composition:

Glucose	104 g

Solution containing the amino acids specified below 200 ml

Pyridoxine	2 mg
Calcium pantothenate	2 mg
Thiamine	30 mg
Riboflavin phosphate	4 mg
Potassium chloride	1.43 g
Sodium bicarbonate	1.50 g
Nicotinamide	50 mg
Monopotassium phosphate	750 mg
Magnesium sulfate	500 mg
Vitamin C	500 mg
Distilled water q.s	500 ml

The amino acid solution employed (Soramin 10%, Darrow Laboratórios S.A.), with 16 g/l nitrogen, contained:

L-phenylalanine	540 mg
L-isoleucine	370 mg
L-leucine	980 mg
L-lysine, acetate	590 mg
L-methionine	530 mg
L-threonine	490 mg
L-tryptophan	180 mg
L-valine	530 mg
L-arginine (base)	1060 mg
L-histidine (base)	460 mg
L-alanine	1030 mg
L-asparagine	380 mg
L-aspartic acid	270 mg
L-glutamic acid	250 mg
L-cystine	30 mg
L-ornithine	260 mg
L-proline	840 mg
L-serine	250 mg
L-tyrosine	160 mg
Glycine	800 mg
Distilled water q.s.	100 ml

The following substances were added to each 100 ml of the parenteral feeding solution: 12.5 units of regular insulin (Biobrás-Bioquímica do Brasil), 0.125 mg glucagon (Eli Lilly), 500 µg folic acid, 6.25 µg vitamin B12, 625 µg zinc sulfate, and 250 mg CMC.

L-triiodothyronine (T3) 2.26 µg/200 g rat weight in ethyl alcohol was placed separately in each syringe containing the solution to prevent T3 precipitation in aqueous medium from interfering with uniform application.

On the 8th day, the animals were sacrificed by ether inhalation after a 24-hour fast and their body and liver weights were recorded. A liver fragment was obtained, weighed immediately, identified and frozen for later DNA measurement.

Animal mortality during the experiment was recorded daily after the beginning of the injection schedule and is reported as percent of the total number of animals in each group.

The variation in liver weight enhancement in group B animals was calculated at the end of the experiment as observed weight minus expected weight divided by expected weight. The latter weight was determined as a function of animal body weight at sacrifice multiplied by the liver weight/body weight ratio factor, defined in group A animals (control). In summary:

$$\text{Variation (\%) in liver weight enhancement} = \frac{\text{Observed weight} - \text{Expected weight}}{\text{Expected weight}} \times 100$$

$$\text{where Expected liver weight} = \text{Body weight at sacrifice} \times \text{factor obtained for group A}$$

Biochemical DNA measurement was performed after trichloroacetic acid extraction using the diphenylamine reaction (3,18).

Statistical analysis: The results are reported as means ± SEM. Data for the two groups were compared by the Mann-Whitney test when necessary, with the level of significance set at p<0.05.

RESULTS

No mortality was observed in group A animals, whereas mortality was 60% in group B, with a biphasic pattern: the first peak occurred during the first two days (35% of all animals) and a second peak occurred during

Table 1
Body and liver weights (g) and liver weight/body weight ratio at the time of sacrifice in group A animals

Body weight	Liver weight	Liver weight/Body weight
227.2	6.064	0.0266
221.2	5.242	0.0236
203.6	5.271	0.0258
202.8	5.625	0.0277
201.2	5.526	0.0274
215	4.990	0.0232
182.5	4.313	0.0236
200.4	5.544	0.0276
194.5	4.655	0.0239
215.4	5.263	0.0244
Mean		0.0254
SEM		± 0.0005

Table 2
Body and liver weights (g), liver weight/body weight ratio and enhancement of hepatic size (%) at the time of sacrifice in group B animals

Body weight	Liver weight	Liver weight/body weight	Enhancement of liver size
241.2	10.793	0.0447	76.17
205.2	10.534	0.0513	102.11
201	10.329	0.0514	102.32
193.5	10.304	0.0533	109.65
205.1	11.199	0.0546	114.97
212.4	11.960	0.0563	121.69
227.4	13.951	0.0614	141.54
200	12.437	0.0622	144.82
Mean		0.0544	114.16
SEM		±0.0020	±7.90

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the last two days (25% of all animals) (Fig. 1). Table 1 shows the body and liver weights of group A animals at the time of sacrifice and their ratio, which was used to calculate the liver weight/body weight index to be used for the determination of expected liver weight in group B animals.

Table 2 shows the individual values for body weight, liver weight, liver weight/body weight ratio and enhancement of hepatic size in group B survivors.

It should be emphasized that two of the animals who died, one on the 6th and the other on the 7th day after the beginning of the injections, having escaped cannibalism on the part of the survivors, could be submitted to autopsy and to the calculation of enhancement of liver size (Table 3).

Table 4 summarizes the results and the statistical comparisons of DNA measurements in animals from the two groups at the time of sacrifice in terms of concentrations in hepatic tissue (mg/g liver) and total DNA/liver (mg).

DISCUSSION

Enhancement of liver size in intact (non-hepatectomized) livers by stimulation with exogenous hepatotrophic factors has been demonstrated in previous studies[19,20] and has been attributed to a regenerative mechanism involving an increase in hepatocyte number.

The major difficulty in the study of increased liver size in experiments in which estimated size is compared to observed mass is to determine estimated size in live animals. This is done indirectly by defining the liver weight/body weight ratio in a group of control animals. This procedure also ties the calculation of liver growth to final body weight, a fact that eliminates the possible influence of treatment with glucose, amino acids and hormones on variations in animal appetite, food consumption and body growth rate[20]. The difference between the mean percent enhancement of liver size obtained in the present study ($114.16 \pm 7.90\%$) and that

Table 3
Body and liver weights (g) and enhancement of liver size (%) of two animals submitted to necropsy.

Day of death	Body weight	Liver weight	Enhancement of liver size
6th	196.9	12.345	146.84
7th	199.2	11.160	120.57

Table 4
DNA concentrations in hepatic tissue (mg/g liver) and total DNA/liver (mg) at the time of sacrifice

Group	DNA concentration	Total DNA
A	2.477 ±0.060	12.99 ±0.46
B	1.765 ±0.120	20.17 ±1.44
	p=0.0005	p=0.0002

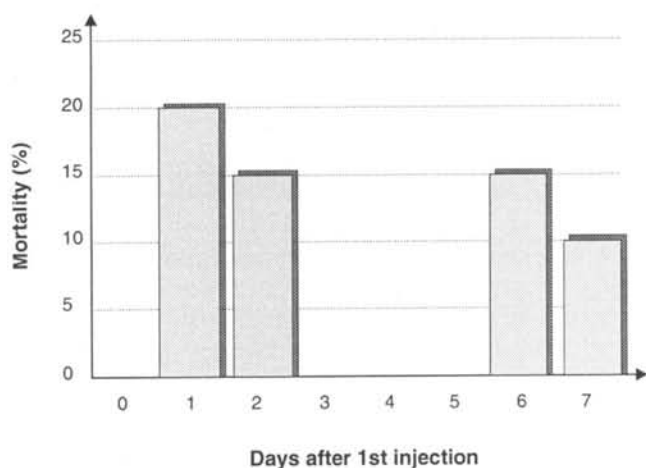**FIGURE 1** - Animal mortality presenting a biphasic profile.

obtained in the previous study (67.54 ± 10.18%) (20) is essentially due to the utilization of a new factor for the liver weight/body weight ratio (0.0254) determined in a specific group of control animals also subjected to intraperitoneal injections of saline solution. The pain and stress of this procedure probably modified through a neuro-hormonal pathway this ratio which had been previously calculated to be 0.033 in intact animals(18). If the enhancement in liver mass obtained in the present study were calculated as a function of the 0.033 factor (calculations not shown), the mean would be 64.84 ± 0.08%, similar to that obtained previously(20). This change definitely provided a control group of greater scientific rigor.

The association of the factors used here with powerful mitogens for hepatocytes such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF), which have proved to be able to induce by themselves an increase in the size of intact livers in animals(6,16), may possibly induce a better response, perhaps within a shorter period of time.

However, the increase in liver growth achieved with the methodological modifications sequentially introduced in our previous studies(19,20) was accompanied by an also increasing mortality rate, with a biphasic behavior(20), i.e., a first peak during the first two days after the beginning of the injections and a second peak during the last two days. This behavior was repeated in the present experiment (Fig. 1). The two peaks were attributed to different causes, the first being related to a possible initial toxicity of the

solutions employed, since it had been observed that a human milk extract added to the solution as the vehicle for growth factors protected the animals(20). This protective action was confirmed by the use of defatted and lyophilized cow's milk, but was accompanied by the onset of non-fatal chemical peritonitis in the animals thus treated (unpublished data). For this reason, in the present study we tried carboxymethylcellulose (CMC) for its protective action, but no effective results were obtained. The administration of the solutions in a more physiological manner by continuous infusion into the portal system, especially in larger animals, into the peritoneal cavity itself and even fractionation of the dose employed into more than one daily intraperitoneal injection may possibly reduce the initial mortality of the experimental animals.

The second mortality peak with an increasing pattern observed during the last days of the previous experiment(20) and also in the present study, was minimized here by the reduction of the number of injection days from 10 to 7, so as to obtain a number of surviving animals whose results could be analyzed statistically without impairment of liver size enhancement. A high rate of liver growth was also observed in the animals that died (Table 3). Studies are currently underway in our laboratory to determine whether this late mortality is related to the increase in liver size, assuming that a maximum limit exists for regenerative liver growth and that this limit was continuously forced by the exogenous hepatotrophic stimulus against the biologically predetermined equilibrium.

The increase in liver size was accompanied by a significant dilution in tissue DNA concentration, as indicated in Table 4. Since the report by HIGGINS & ANDERSON(11), it has been known that the parenchyma gains fluid during the late stages of regeneration. Such a great increase in liver size, although accompanied by some degree of variation in tissue composition, does not seem to invalidate our interpretation that its cause is mainly the formation of new cells, in view of the great and significant increase in total DNA mass per liver (from 12.99 mg to 20.17 mg, p = 0.0002). A study of the variations in tissue composition may better elucidate this detail.

The difficulty in maintaining the portal vein catheterized for one week in small animals was obviated by applying the solutions under study intraperitoneally, although accepting some systemic absorption through the abdominal wall. It has been demonstrated that exogenous hepatotrophic factors have the highest effect when introduced through the portal vein, mimicking their physiological route, when compared to administration through a peripheral vein(19). We consider this observation

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to be very important although it has not been considered before in studies aiming at the treatment of certain liver diseases with the administration of exogenous factors as a regenerative stimulus(1,5,8,15,23).

We believe that the perspective of stimulating liver regeneration by the administration of hepatotrophic factors by the portal route has great clinical applicability both in situations of reduced liver size after hepatectomy or hepatocyte necrosis (hepatitis and abscesses) and in situations involving the intact liver, such as cases of transplants from live donors, providing the recipient with a greater implantable hepatic segment and the donor with a greater residual liver mass. Also, patients with cirrhosis, in whom regenerative stimulation by partial hepatectomy has been shown to produce a histological and functional improvement(4,9,10,12,13,17,22) may benefit from this method without the inconvenience of reduced function brought about by hepatectomy.

Resumo

Objetivos: Confirmar observações anteriores (PARRA e col. [19,20]) que sugeriam a possibilidade de fazer crescer o fígado em animais intactos, por estímulo hepatotrófico exógeno, ultrapassando seu tamanho biologicamente pré-determinado. **Material e Métodos**: Dois grupos de fêmeas de ratos Wistar, foram injetadas diariamente na cavidade peritoneal (40 ml/kg) por sete dias consecutivos com as seguintes soluções: GRUPO A (controle) - solução salina com carboximetilcelulose (CMC) a 0.25%; GRUPO B - solução de fatores hepatotróficos exógenos constituída de glicose, aminoácidos, insulina, glucagon, vitamins, eletrólitos, triiodotironina e CMC na mesma concentração do grupo controle. No oitavo dia os animais foram sacrificados e avaliados os seguintes parâmetros: variação da massa hepática e DNA total do fígado dos animais do grupo experimental em relação ao grupo controle. **Resultados**: Foi observado crescimento da massa hepática de 114,16 ± 7,9% acima do esperado e aumento do DNA total hepático de 12,99 ± 0,46 mg para 20,17 por fígado (p=0.0002). **Conclusão**: O aumento do tamanho do fígado a partir de sua massa primitiva, estimulada por fatores hepatotróficos exógenos, pode vir a ter aplicações em hepatologia, como em casos de cirroses revertendo parcialmente as alterações histológicas e funcionais, ou em transplantes intre-vivos, criando maior massa hepática a ser dividida entre doador e receptor.

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