The Effect of Hepatic Stimulator Substance (HSS) on Liver Regeneration Arrest Induced by 5-HT$_2$ Receptor Blockade

KONSTANTINOS N. TZIROGIANNIS[1], GEORGIOS I. PANOUTSOPOULOS[1], GEORGE K. PAPADIMAS[1], MARIA D. DEMONAKOU[2], ROSA I. HERETI[1], GEORGIA A. MANTA[1], KALLIOPI T. KOURENTZI[1], VASILIKI G. KONDILI[1], KATERINA N. ALEXANDROPOULOU[1] and MICHAEL G. MYKONIATIS[1]

[1]*Department of Experimental Pharmacology, Medical School, Athens University, 75 Mikras Asias Street, Athens 115 27;*
[2]*Histopathology Laboratory, Sismanoglion G.D. Hospital, Sismanogliou 1, Marousi, Attiki 151 27, Greece*

Abstract. *Background: The restorative effect of hepatic stimulator substance (HSS) against hepatic regeneration arrest induced by 5-HT$_2$ receptor blockade was investigated. Materials and Methods: Male Wistar rats were subjected to 60-70% partial hepatectomy and to 5-HT$_2$ receptor blockade at 16 h after partial hepatectomy by ketanserin administration (6 mg/kg bodyweight intraperitoneally; group I). HSS at the dose of 100 mg protein/kg bodyweight was administered at 10 or 17 h after partial hepatectomy in ketanserin-treated rats (groups II and III). The mitotic index in hematoxylin-eosin-stained liver sections, immunochemical detection of PCNA and Ki 67 nuclear antigens and the rate of [^{3}H]-thymidine incorporation into hepatic DNA were used as indices of liver regeneration. Results: Liver regeneration, as evaluated by [^{3}H]-thymidine incorporation into hepatic DNA, mitotic index, PCNA and Ki67 nuclear antigens, peaked at 40 h in groups I, II and III of rats and no significant differences were observed between the studied groups. Conclusion: HSS administration is not capable of reversing the liver regeneration arrest induced by 5-HT$_2$ receptor blockade.*

Serotonin is implicated in a great variety of physiological pathways and disease processes that by far outnumber those of any other neurotransmitter (1) and mediates its actions through at least seven distinct types of receptors (2). The role of serotonin in liver regeneration after partial hepatectomy has recently been studied in cultured rat hepatocytes, where serotonin induced a dose-dependent increase in DNA synthesis in the presence of endothelial growth factor (EGF), which implied a role of serotonin as a cofactor during DNA synthesis (3).

The 5-HT$_2$ receptor is the most abundant receptor type in the liver, which operates through the increase of intracellular calcium levels by phosphatidyloinositol (PI) hydrolysis (4, 5). This type of receptor is implicated in cell proliferation in the central nervous system (1) and in the periphery (6-8) where apoptosis (9, 10), neuronal toxicity (11), cell survival (10) and many other actions are reversed by specific inhibitors of the 5-HT$_2$ receptor system (1).

Ketanserin is a 5-HT$_2$ receptor antagonist with relatively weaker α1-adrenoreceptor and weaker α_1-adrenoreceptor and H$_1$-receptor blocking properties (12, 13), which has been introduced as an antihypertensive agent, as well as for the treatment of vasospastic disorders (14). Ketanserin has the ability to reverse many of the physiological actions of serotonin mediated through the 5-HT$_2$ receptor type and, in addition to its vasoactive properties, it also blocks platelet aggregation and improves hemorrheological properties (14).

Hepatic stimulator substance (HSS) is a liver-specific, species non-specific growth factor, which is present in the liver of weanling rats and in the regenerating liver of adult rats. The factor is a potent stimulator of liver regeneration when administered intraperitoneally in partially hepatectomized (30-34%) rats (15, 16). HSS also has the capability of protecting the liver from chemical injury induced by several poisons and drugs including cadmium (17-19). The peptide has been renamed by other researchers as the augmenter of liver regeneration (ALR) (20) and the isolation and cloning of the molecule has been achieved (21). The c-DNA for rat ALR encodes a protein of 198 amino acid residues and has a molecular weight of about 22 kD. The molecule is highly conserved in humans and rats and the genes are mapped in chromosomes 16 and 17, respectively (22). The discrepancy between the two different names still stands in the bibliography, although in the present study the macromolecule is referred to as HSS.

Correspondence to: Dr Georgios I. Panoutsopoulos, 5 Tenedou Street, Platia Amerikis, Athens 112 57, Greece. Tel: +30210-8649617, Fax: +30210-7462554, e-mail: geopanou@otenet.gr

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The purpose of this study was to examine the effect of HSS administration on liver regeneration arrest induced by 5-HT$_2$ receptor blockade.

Materials and Methods

Experimental animal model. Male Wistar rats (160-180 g), four to five months old, obtained from the Hellenic Pasteur Institute (Athens, Greece), were used in this study. The animals were kept in a temperature-controlled room (22-24˚C), under 12 h of light (08.00-20.00 h) and 12 h of darkness (20.00-08.00 h). All experiments were conducted between 07.00-09.00 h with the animals under light ether anesthesia. Serotonin receptor 2 blockade was achieved by intraperitoneal administration of ketanserin (Janssen Biotech N.V., Olen, Belgium).

The experimental rats were randomly selected and assigned to groups as follows:
Group I: rats submitted to 60-70% partial hepatectomy and intraperitoneal administration of ketanserin (6 mg/kg bodyweight) at 16 h after partial hepatectomy.
Group II: rats submitted to 60-70% partial hepatectomy, intraperitoneal administration of ketanserin as in group I and intraperitoneal administration of HSS (100 mg protein/kg bodyweight) at 10 h after partial hepatectomy.
Group III: rats submitted to 60-70% partial hepatectomy, intraperitoneal administration of ketanserin as in group I and intraperitoneal administration of HSS (100 mg protein/kg bodyweight) at 17 h after partial hepatectomy.

Animals from all groups were killed at 20, 24, 32, 40, 48 and 60 h after partial hepatectomy. One hour prior to sacrifice, the animals of all groups were injected with [3H]-thymidine at the dose of 250 μCi/kg bodyweight intraperitoneally. A standard portion of the median liver lobe was used for histological evaluation and the rest of the liver was rapidly frozen in liquid nitrogen for further determinations.

Histological evaluation. A standard portion of the liver was fixed in 4% buffered-formalin for 24 h. Sections, 5 μm thick, were processed routinely, hematoxylin-eosin (HE)-stained and analyzed for mitoses. Mitoses were counted in 10 randomly selected high-power fields (HPF) and expressed as the mean number of mitoses/1000 liver cells.

The mitotic index was also evaluated by the immunochemical detection of PCNA (monoclonal mouse anti-proliferating cell nuclear antigen, PC 10 clone, 1:200; DakoCytomation, Glostrup, Denmark) and Ki 67 nuclear antigens (MIB 5 clone, 1:50 with microwave pre-treatment; DakoCytomation) and expressed per 1000 liver cells.

Liver regeneration. The rate of liver regeneration was evaluated by the rate of [3H]-thymidine incorporation into hepatic DNA, by the mitotic index in HE sections and the immunochemical detection of PCNA and Ki 67 nuclear antigens.

Rate of [3H]-thymidine incorporation into hepatic DNA. The animals of all groups were injected intraperitoneally with 25 μCi/100 g bodyweight of [3H]-thymidine 1 h prior to sacrifice. DNA was extracted from the tissue according to the method of Munro and Fleck (23). The content of tissue DNA was estimated by the method of Richards (24). The rate of [3H]-thymidine incorporation

into hepatic DNA was calculated from the radioactivity measured in a liquid scintillation counter (Wallac LKB 1211, Rackbeta, Sweden) and the results were expressed as counts/min/μg of DNA.

Preparation of HSS. HSS was extracted and partially purified following the method of LaBrecque *et al.* (25) and Fleig *et al.* (26). Briefly, α 35% (w/v) homogenate of 1 g of liver in 0.9% NaCl was prepared using a Tri-R Stir-R Model S63C variable speed homogenizer fitted with a Teflon pestle. The homogenate was incubated at 65˚C in a water bath for 15 min and centrifuged at 27,000 x g for 20 min at 4˚C. Six volumes of ice-cold absolute ethanol were added to the supernatant and stirred for 2 h at 4˚C. After centrifugation at 27,000 x g for 20 min, the precipitate was dissolved in water followed by another centrifugation at 27,000 x g for 20 min. The supernatant was lyophilized and stored at –30˚C and its biological activity was tested using the bioassay described below.

Bioassay of HSS activity. The determination of HSS activity was performed using male Wistar rats weighing 80-100 g. The animals were subjected to partial hepatectomy (30-34%) by resection of the left lateral lobe of the liver. The animals were injected intraperitoneally with 100 mg/kg bodyweight of HSS (lyophilized HSS was dissolved in 0.9% NaCl) 12 h after partial hepatectomy. The animals were sacrificed 12 h after the HSS administration and 1 h prior to sacrifice they were injected with [3H]-thymidine (250 μCi/kg bodyweight) and the livers were excised and rapidly frozen at –80˚C. The HSS biological activity was estimated by [3H]-thymidine incorporation into hepatic DNA. The administration of HSS at the dose of 100 mg/kg bodyweight resulted in doubling the [3H]-thymidine incorporation into hepatic DNA in relation to the control group of 30-34% partially hepatectomized rats.

Statistical analysis. The data are expressed as mean±SE. All observations were obtained from at least six animals, and the assays were done in duplicates. The statistical analysis of the results was performed by one-way analysis of variance and unpaired Student's *t*-test.

Results

In group I rats, ketanserin administration at 16 h after partial hepatectomy arrested liver regeneration at 20 and 24 h after partial hepatectomy as evaluated by [3H]-thymidine incorporation into hepatic DNA (Figure 1). Liver regeneration in ketanserin-treated rats started increasing at 24 h and remained at relatively high levels between 32 and 60 h (Figure 1). HSS administration at 10 and 17 h after partial hepatectomy did not alter the time-pattern of liver regeneration and the regenerative activity was almost identical for groups I, II and III (Figure 1), without statistically significant differences between the three groups of rats.

The mitotic index in HE-stained sections peaked at 40 h for all groups of rats, with high levels being observed at 32 and 48 h (Figure 2). The immunochemical detection of PCNA started to increase gradually from 20 h to 40 h, where it peaked. Subsequently, it decreased at 48 h and starting increasing again at 60 h for all groups of rats
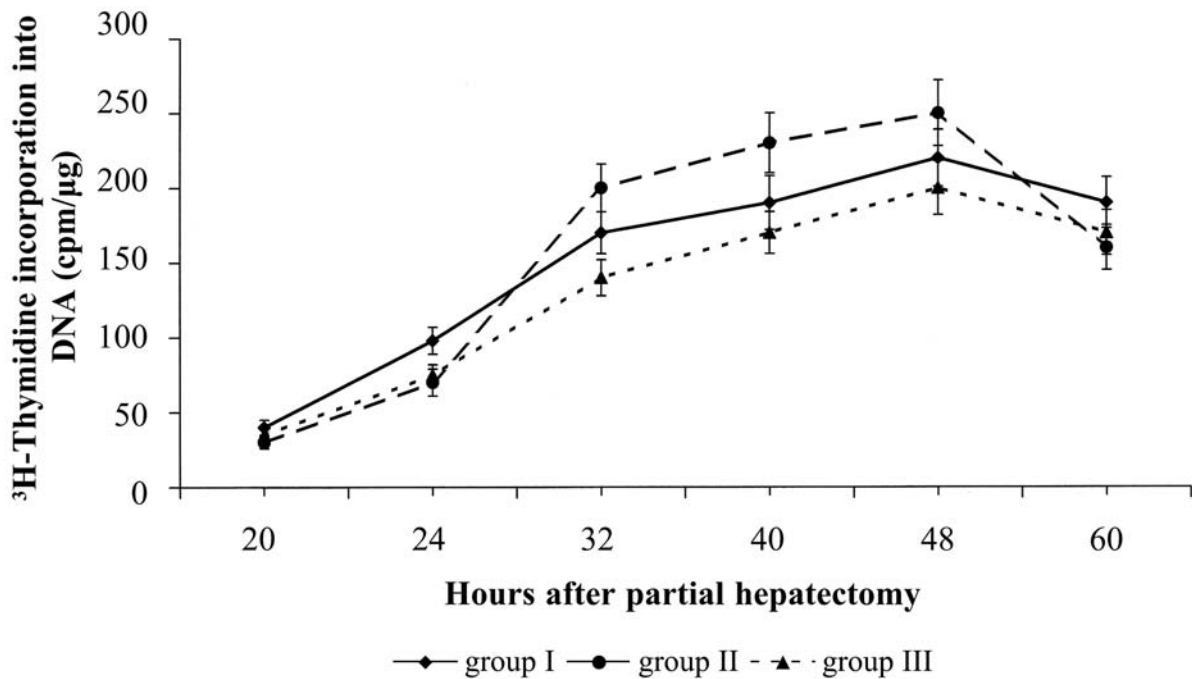
Figure 1. *Rate of ^3H-thymidine incorporation into hepatic DNA in 60-70% partially hepatectomized rats having received ketanserin at 16 h (6 mg/kg bodyweight) (group I), or ketanserin at 16 h and HSS (100 mg protein/kg bodyweight) 10 h after partial hepatectomy (group II), or ketanserin at 16 h and HSS (100 mg protein/kg bodyweight) 17 h after partial hepatectomy (group III). Results represent the findings of six rats killed at all time-points examined. Values are expressed as means±SE.*

(Figures 3, 4a and 4b). No statistically significant differences were observed between the three groups of rats. The number of Ki67 nuclear antigen-positive cells also showed the same time-pattern for all three groups of rats and it was similar to the time-pattern of PCNA-positive cells for all time-points examined (data not shown).

Discussion

Liver regeneration has been a field of intense scientific research in recent decades, but the mechanisms that control it are still obscure (27, 28). Numerous chemically diverse endocrine, paracrine and autocrine factors influence liver regeneration and their numbers are still increasing. Among these, the role of neurotransmitters has been investigated thoroughly only in the case of catecholamines, especially norepinephrine, while less is known about the others (29, 30).

The role of serotonin on liver regeneration has recently started to emerge and it is still poorly understood. The monoamine has been found to induce a dose-dependent increase in DNA synthesis in cultured rat hepatocytes, but the presence of EGF was an absolute prerequisite for this effect (3). From previous results of our research group on the possible role of serotonin in liver regeneration after

partial hepatectomy in the rat liver, it was clear that the activation of 5-HT$_2$ receptors is an important event since blockade of this receptor type close to the G1/S transition point arrested liver regeneration (work submitted for publication). Liver serotonin levels do not appear to increase substantially during hepatic DNA synthesis, but it seems that the susceptibility of hepatocytes to the action of serotonin is increased (work submitted for publication). However, the role of the other serotoninergic receptor types on liver regeneration is still unknown.

In recent years, scientific interest has been focused on the cell-cycle progression of liver cells in the regenerating liver and on the various checkpoints that operate at the molecular level. The role of growth factors has been intensively investigated and, according to current knowledge, they seem to control the G1/S transition checkpoint, which is the most important checkpoint in the G1-phase. The various growth factors appear to operate in a convergent pathway at the G1/S transition through the activation of Ras/mitogen-activated protein kinase (MAPK). Following the activation of MAPK, cyclin D1 protein is expressed, which drives hepatocytes in the S-phase of the cell-cycle (31, 32).

HSS has been found to protect the liver against the toxic effects of various poisons and to restore the hepatic
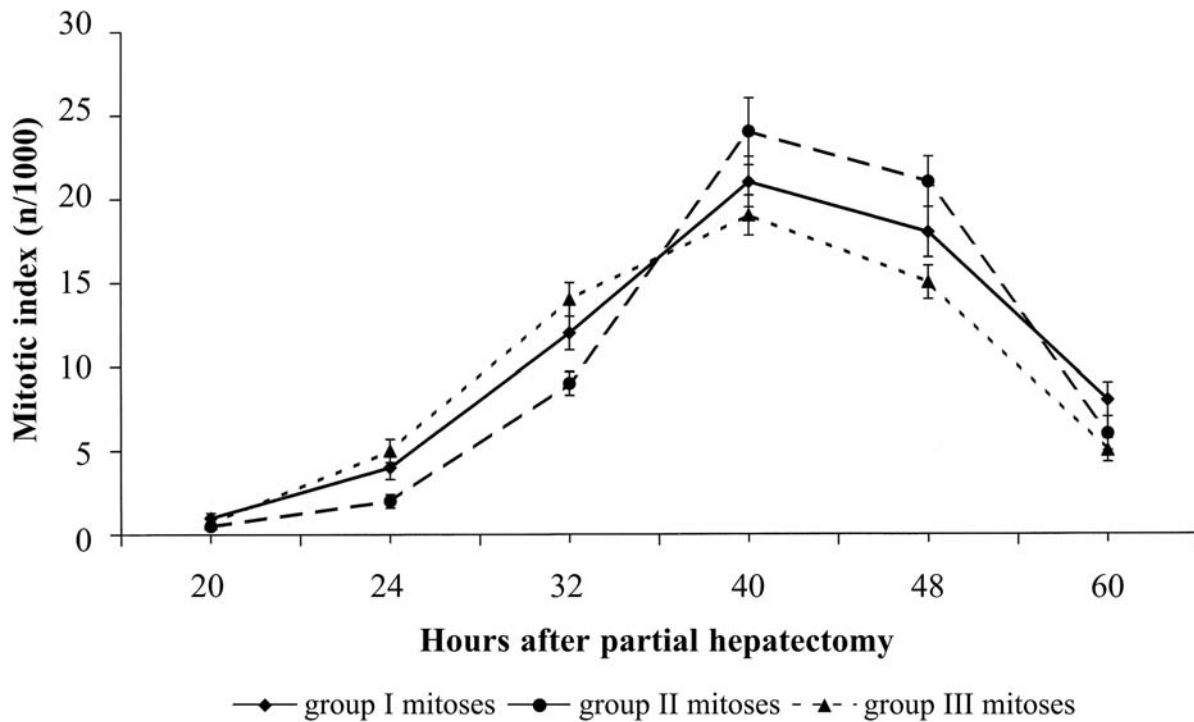
Figure 2. *Time-course of mitotic index (HE sections) in 60-70% partially hepatectomized rats having received ketanserin at 16 h (6 mg/kg bodyweight) (group I), or ketanserin at 16 h and HSS (100 mg protein/kg bodyweight) 10 h after partial hepatectomy (group II), or ketanserin at 16 h and HSS (100 mg protein/kg bodyweight) 17 h after partial hepatectomy (group III). Results represent the findings of six rats killed at all time-points examined. Values are expressed as means±SE.*

regenerative capacity when administered intraperitoneally. The levels of HSS biological activity have also been found to increase around the G1/S transition point (33, 34), which hints at a possible participation of this factor among the network of factors that control the G1/S transition point.

In the present study, HSS administration was found ineffective in restoring the suppressed hepatic regenerative activity induced by serotonin receptor blockade. Serotonin, through activation of 5-HT$_2$ receptors, may act as a cofactor of DNA synthesis or participate in the G1/S transition checkpoint. Therefore, it seems that the activation of the serotoninergic receptors constitutes another necessary subset of required factors which are totally distinct from those dependent on HSS, or possibly on the other growth factors.

The role of neurotransmitters in liver regeneration has been thoroughly studied only in the case of catecholamines, especially norepinephrine, although not fully elucidated. It has been previously shown that norepineprhine stimulates DNA synthesis only when EGF is present (through the a_1-adrenergic receptor) and the mechanism of action is related to the regulation of EGF receptors, and/or EGF protein levels (29, 30), as well as the offsetting of mitoinhibitory stimuli such as TGF-β1 (30). Catecholamine

levels also increase in the first few hours after partial hepatectomy and this temporal pattern is quite distinct from that observed by our research group for serotonin.

Therefore, it seems that another pattern for peripheral neurotransmitting action emerges for serotonin in relation to liver regeneration, that may be quite distinct from that reported in the literature. Possible interactions *in vivo* between serotoninergic activity and growth factors cannot be excluded, since serotonin acted as a cofactor for DNA synthesis, in the presence of EGF, in experiments with cultured hepatocytes (3). Further research is needed in order to fully elucidate this pathway.

References

1 Azmitia EC: Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation and apoptosis. Brain Res Bull *56*: 413-424, 2001.

2 Barnes NM and Sharp T: A review of central 5-HT receptors and their function. Neuropharmacology *38*: 1083-1152, 1999.

3 Balasubramanian C and Paulose CS: Induction of DNA synthesis in primary cultures of rat hepatocytes by serotonin: possible involvement of serotonin S2 receptor. Hepatology *27*: 62-66, 1998.
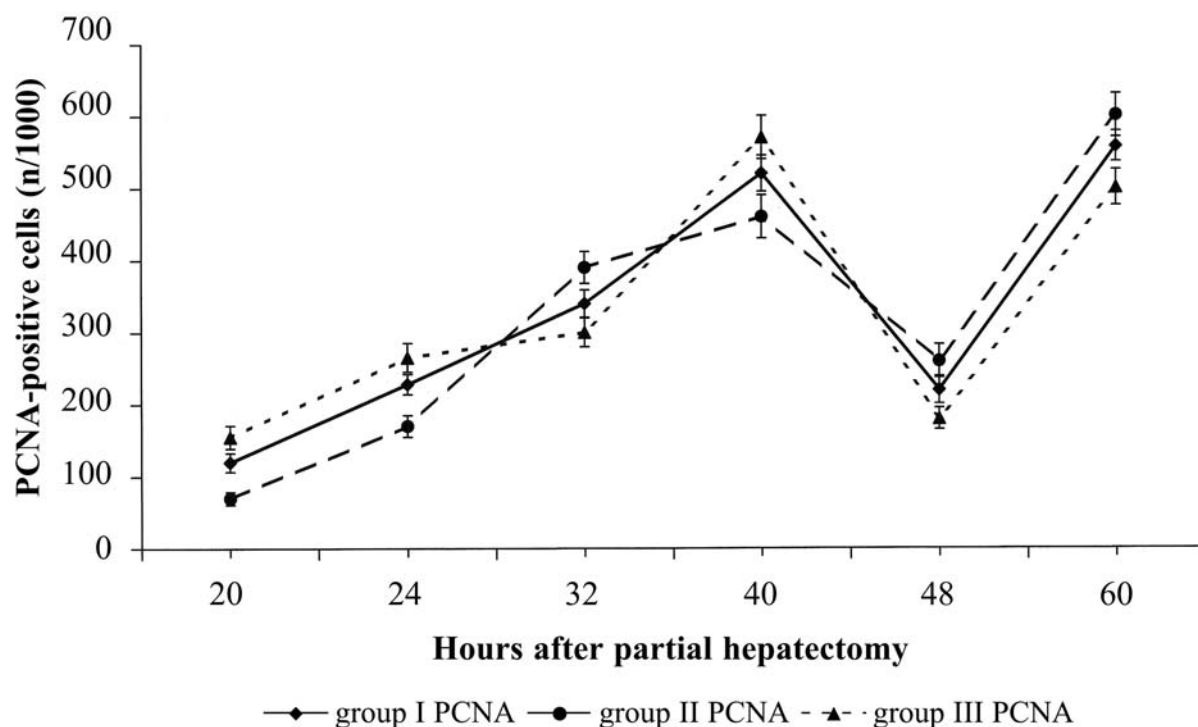
Figure 3. *Time-course of PCNA-positive cells in 60-70% partially hepatectomized rats having received ketanserin at 16 h (6 mg/kg bodyweight) (group I), or ketanserin at 16 h and HSS (100 mg protein/kg bodyweight) 10 h after partial hepatectomy (group II), or ketanserin at 16 h and HSS (100 mg protein/kg bodyweight) 17 h after partial hepatectomy (group III). Results represent the findings of six rats killed at all time-points examined. Values are expressed as means±SE.*

4 Adayev T, El-Sherif Y, Barua M, Penington NJ and Banerjee P: Agonist stimulation of the serotonin 1A receptor causes suppression of anoxia-induced apoptosis *via* mitogen-activated protein kinase in neuronal HN2-5 cells. J Neurochem *72*: 1489-1496, 1999.

5 Lucaites VL, Nelson DL, Wainscott DB and Baez M: Receptor subtype and density determine the coupling repertoire of the 5-HT$_2$ receptor subfamily. Life Sci *59*: 1081-1095, 1996.

6 Pakala R, Willerson JT and Benedict CR: Mitogenic effect of serotonin on vascular endothelial cells. Circulation *90*: 1919-1926, 1994.

7 Massey MF, Davies MG, Svendsen E, Klyachkin ML, Schwartz LB, Barber L, McCann RL and Hagen PO: Reduction of experimental vein graft intimal hyperplasia by ketanserin. J Surg Res *54*: 530-538, 1993.

8 Cirillo P, Golino P, Ragni M, Battaglia C, Pacifico F, Formisano S, Buono C, Condoreli M and Chiariello M: Activated platelets and leucocytes cooperatively stimulate smooth muscle cell proliferation and proto-oncogene expression *via* release of soluble growth factors. Cardiovasc Res *43*: 210-218, 1999.

9 Ozaki S, DeWald DB, Shope JC, Chen J and Prestwich GD: Intracellular delivery of phosphoinositides and inositol phosphates using polyamine carriers. Proc Natl Acad Sci USA *97*: 11286-11291, 2000.

10 Hajnoczky G, Csordas G, Madesh M and Pacher P: Control of apoptosis by IP(3) and ryanodine receptor driven calcium signals. Cell Calcium *28*: 349-363, 2000.

11 Schmidt CJ, Black CK and Taylor VL: Antagonism of the neurotoxicity due to a single administration of methylenedi-oxymethamphetamine. Eur J Pharmacol *181*: 59-70, 1990.

12 Brogden RN and Sorkin EM: Ketanserin: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in hypertension and peripheral vascular disease. Drugs *40*: 903-949, 1990.

13 Leysen JE, Awouters F, Kennis L, Laduron PM, Vandenberk J and Janssen PA: Receptor binding profile of R41468, a novel antagonist at 5-HT$_2$ receptors. Life Sci *28*: 1015-1022, 1981.

14 Persson B, Heykants J and Hedner T: Clinical pharmacokinetics of ketanserin. Clin Pharmacokinet *20*: 263-279, 1991.

15 LaBrecque DR and Pesch LA: Preparation and partial characterization of hepatic regenerative stimulatory substance (SS) from rat liver. J Physiol *248*: 273-284, 1975.

16 Francavilla A, Ove P, Polimero L, Coetzee M, Makowka L, Rose J, Van Thiel DH and Starzl TE: Extraction and partial purification of a hepatic stimulatory substance in rats, mice, and dogs. Cancer Res *47*: 5600-5605, 1987.

17 Mei MH, An W, Zhang BH, Shao Q and Gong DZ: Hepatic stimulator substance protects against acute liver failure induced by carbon tetrachloride poisoning in mice. Hepatology *17*: 638-644, 1993.

18 An W and Mei MH: Protective role of hepatic stimulator substance against experimental acute liver failure in mice. Sheng Li Xue Bao *43*: 415-427, 1991.
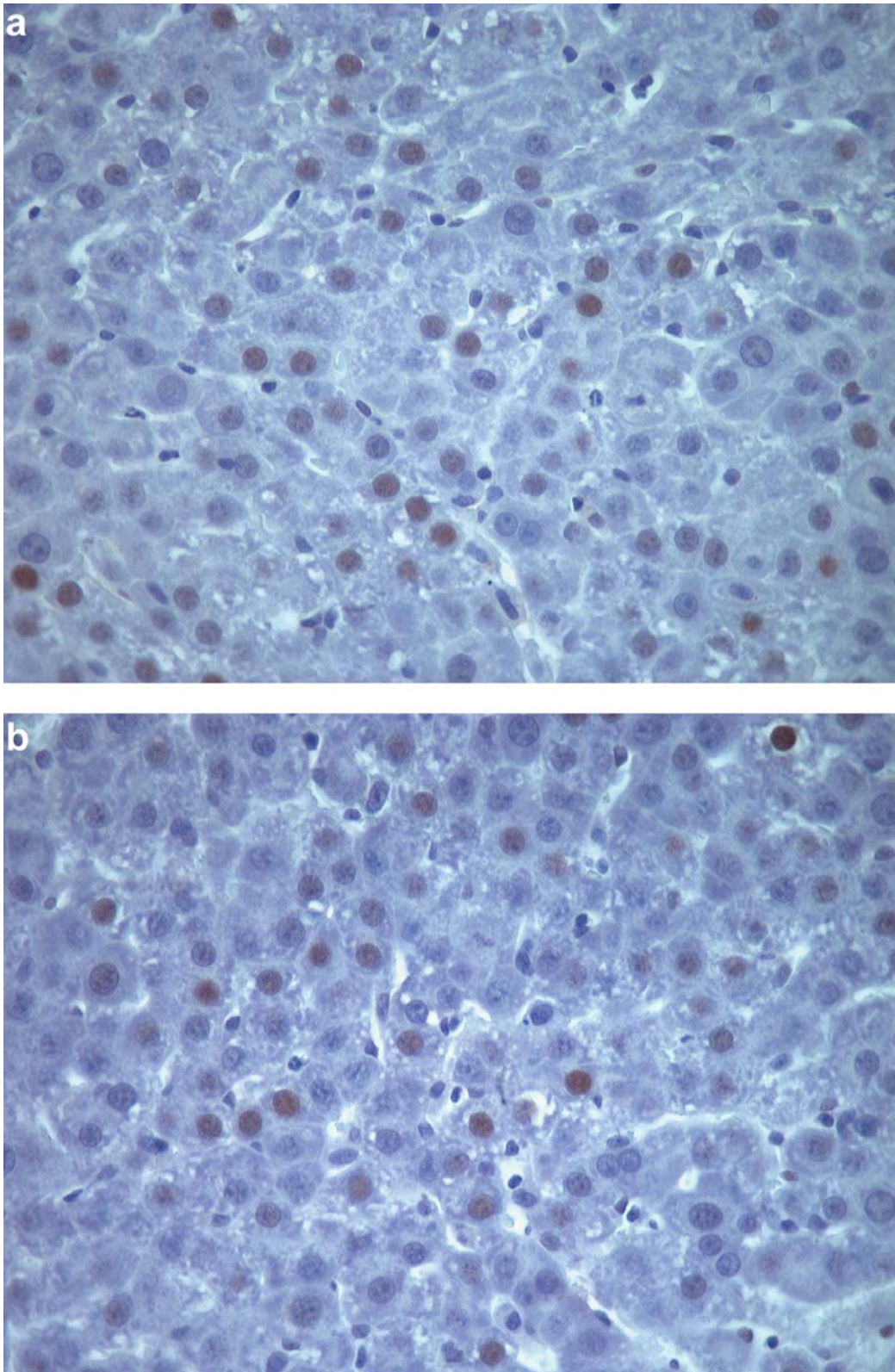
Figure 4. *a. PCNA-positive cells at 40 h (x400) in 60-70% partially hepatectomized rats having received ketanserin (6 mg/kg bodyweight) at 16 h (group I). b. PCNA-positive cells at 40 h (x400) in 60-70% partially hepatectomized rats having received ketanserin at 16 h (6 mg/kg bodyweight) and HSS (100 mg protein/kg bodyweight) at 10 h after partial hepatectomy (group II).*

19 Theocharis SE, Margeli AP, Agapitos EV, Mykoniatis MG, Kittas CN and Davaris PS: Effect of hepatic stimulator substance administration on tissue regeneration due to thioacetamide-induced liver injury in rats. Scan J Gastroenterol *33*: 656-663, 1998.

20 Francavilla A, Hagiya M, Porter KA, Polimero L, Ihara I and Starzl TE: Augmenter of liver regeneration : its place in the universe of hepatic growth factors. Hepatology *20*: 747-757, 1994.

21 Hagiya M, Francavilla A, Polimero L, Ihara I, Sakai H, Seki T, Simonishi M, Porter KA and Starzl TE: Cloning and sequence analysis of the rat augmenter of liver regeneration (ALR) gene: expression of biologicaly active recombinant ALR and demonstration of tissue distribution. Proc Natl Acad Sci USA *92*: 3076, 1995.

22 Giorda R, Hagiya M, Seki T, Simonishi M, Sakai H, Michaelson J, Francavilla A, Starzl TE and Trucco M: Analysis of the structure and expression of the augmenter of liver regeneration (ALR) gene. Mol Med *2*: 97-108, 1996.

23 Munro HN and Fleck A: Recent developments in the measurement of nucleic acids in biological materials. Analyst *91*: 78-88, 1966.

24 Richards GM: Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal Biochem *57*: 369-376, 1974.

25 LaBrecque DR and Bachur NR: Hepatic stimulator substance: physicochemical characteristics and specificity. Am J Physiol *242*: G281-G288, 1982.

26 Fleig WE, Lehmann H, Wagner H, Hoss G and Ditschuneit H: Hepatic regenerative stimulator substance in the rabbit: relation to liver regeneration after partial hepatectomy. J Hepatol *3*: 19-26, 1986.

27 Fausto N: Liver regeneration. J Hepatol *32*: 19-31, 2000.

28 Mangnall D, Bird NC and Majeed AW: The molecular physiology of liver regeneration following partial hepatectomy. Liver Inter *23*: 124-138, 2003.

29 Cruise JL, Houck KA and Michalopoulos G: Early events in the regulation of hepatocyte DNA synthesis: the role of alpha-adrenergic stimulation. Scand J Gastroenterol Suppl *151*: 19-30, 1988.

30 Houck KA, Cruise JL and Michalopoulos G: Norepinephrine modulates the growth-inhibitory effect of transforming growth factor- beta in primary rat hepatocyte cultures. J Cell Physiol *135*: 551-555, 1988.

31 Talarmin H, Rescan C, Cariou S, Glaise D, Zanninelli G, Bilodeau M, Loyer P, Guguen-Guillouzo C and Baffert G: The mitogen-activated protein kinase/ extracellular signal-regulated kinase cascade activation is a key signaling pathway involved in the regulation of G1 phase progression in proliferating hepatocytes. Mol Cell Biol *19*: 6003-6011, 1999.

32 Albrecht JH and Hansen LK: Cyclin D1 promotes mitogen-independent cell cycle progression in hepatocytes. Cell Growth Differ *10*: 397-404, 1999.

33 Kondili VG, Tzirogiannis KN, Androutsos CD, Papadimas GK, Demonakou MD, Hereti RI, Manta GA, Kourentzi KT, Triantaphyllou MI and Panoutsopoulos GI: The hepatoprotective effect of Hepatic Stimulator Substance (HSS) against liver regeneration arrest induced by acute ethanol intoxication. Dig Dis Sci *50*: 297-307, 2005.

34 Tzirogiannis KN, Papadimas GK, Kourenzi KT, Kondyli VG, Androutsos CD, Hereti RI, Triantaphyllou MI and Panoutsopoulos GI: The role of hepatic stimulator substance (HSS) on liver regeneration arrest induced by cadmium. In Vivo *19*: 695-704, 2005.