Papers Published


Abstracts Presented

1. **Shilpa Joy**, Nandhu M. S. & C. S. Paulose. GABA - Chitosan nanoparticles induced hepatocyte proliferation in partially hepatectomised


Evaluation of GABA-Chitosan Nanoparticle Induced Cell Signaling Activation During Liver Regeneration After Partial Hepatectomy

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Liver damage due to infection, cirrhosis, accidents and diseases lead to destruction of hepatocytes and their regeneration to its original form is important for the proper functioning of the body. Gamma aminobutyric acid (GABA), a neurotransmitter, was coupled with a biopolymer chitosan and the nanosized complexes were made. The morphology was studied by scanning electron microscope and the interaction of GABA with chitosan was analysed by FT-IR spectroscopy. The interaction of GABA-chitosan nanoparticles with hepatocytes were observed by FITC labeled nanoparticles. After partial hepatectomy in male Wistar rats, DNA synthesis was estimated by tritiated thymidine uptake and the activity of thymidine kinase and protein synthesis by tritiated leucine uptake in hepatocytes. There was an increase in tritiated thymidine uptake in partially hepatectomised groups with nanoparticle treatment (GCNP) when compared to partially hepatectomised groups without nanoparticle treatment (PHNT) and with pure GABA treatment (G). Inositol 1,4,5 trisphosphate (IP$_3$) content and gene expression of phospholipase C mRNA and nuclear factor kappa-light-chain-enhancer of activated B (NF-$\kappa$B) mRNA was decreased for groups G and GCNP with respect to PHNT. Thus our results showed increased hepatocyte regeneration with decreased cell death in group G and more better with GCNP when compared to PHNT.

Keywords: Biocompatible Material, Chitosan, GABA, Hepatocyte Proliferation, Nanoparticles, Cell Division.

1. INTRODUCTION

Nanoparticulate drug delivery systems provide wide opportunities for solving problems associated with drug stability or disease states and create great expectations in the area of drug delivery. Nanotechnology, in a simple way, explains the technology that deals with one billionth of a meter scale. Fewer side effects, poor bioavailability, absorption at intestine, solubility, specific delivery to site of action with good pharmacological efficiency, slow release, degradation of drug and effective therapeutic outcome, are the major challenges faced by most of the drug delivery systems. To a great extend, biopolymer coated drug delivery systems coupled with nanotechnology alleviate the major drawbacks of the common delivery methods. Chitosan, deacetylated chitin, is a copolymer of $\beta$-(1,4) linked glucosamine (deacetylated unit) and $N$-acetyl glucosamine (acetylated unit). Chitosan is biodegradable, non-toxic and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections. To deliver drugs directly to the intended site of action and to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs, polymeric drug delivery systems can be used.

Gamma amino butyric acid (GABA) is a non proteinacious amino acid and is an important inhibitory neurotransmitter in the vertebrate central nervous system. Apart from the inhibitory role, it is reported that GABA involves in the cell proliferation in different regions of the body. The proliferative role of GABA was observed in the development of outer retina in rabbits, TM3 Leydig cell multiplication in testis and promotes neurite growth, cell proliferation and migration. Baclofen, a GABA agonist, induced EGF mediated DNA synthesis in hepatocyte in vitro. There is an increase in hepatocyte proliferation through the activation of GABA$_\beta$ receptor. Also, it significantly reduced the TGF $\beta$1 suppression of EGF induced DNA synthesis. Thus the activation of GABA receptors, trigger DNA synthesis,
mediated through the G protein, in primary cultures of rat hepatocytes. The expression of the stimulatory and inhibitory α-subunit of G proteins coupled receptors to the effector targets like adenylyl cyclase cause biphasic increase in hepatic cAMP. The cell proliferation is initialized by the activation of cAMP regulated transcription factors and phosphorylation of cAMP regulatory element binding protein which influence the induction of cAMP inducible genes in the regenerating liver. GABA is synthesized from glutamate, which is decarboxylated by glutamate decarboxylase (GAD). The major catabolic route for GABA is transamination with alpha-ketoglutarate catalyzed by GABA transaminase. Succinic semialdehyde formed in this reaction is rapidly oxidized to succinate by succinic semialdehyde dehydrogenase. This is the major pathway of GABA metabolism and is termed the GABA shunt. The succinate so formed is utilized in citrate cycle and promote the energy production.

The reduction in apoptosis enhances the regeneration of cells. The signals evoked by IP3 mediated Ca2+ ions in mitochondria triggers apoptosis. Phospholipase C (PLC) is the enzyme involved in the synthesis of IP3 and thus the increased level of IP3 and Phospholipase C result in enhanced apoptosis. The triggering of protein kinase C occurs through IP3 mediated signaling pathway, which further leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB activates TNF-α mediated cell death. Thus the suppression of NF-κB promotes liver regeneration. Apoptosis was further studied by focusing the expression of Caspase-8 gene. Nanoparticles of GABA entrapped in chitosan matrix were prepared for the delivery of GABA to partially hepatectomized liver. Normally the uptake of GABA from the serum by hepatocytes are occurred by a sodium dependent transporter system and the metabolism of GABA by GABA transaminase (GABA-T), which is primarily associated with both plasma and mitochondrial membranes. Therefore, the present study was undertaken to investigate the fastened tissue regeneration efficiency of GABA in a pharmacologically efficient way in partially hepatectomised rat model which certainly explain the therapeutic possibilities of GABA—chitosan nanoparticles for hepatocyte regeneration associated biochemical alterations in liver.

2. EXPERIMENTAL DETAILS

2.1. Chemicals Used and Their Sources

Biochemicals, Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [3H]Gamma amino butyric acid (Sp. Activity 76.2 Ci/mol), [3H] thymidine (Sp. Activity 18.0 Ci/mol) and [3H] leucine (Sp. Activity 63.0 Ci/mol) was purchased from Amersham Life Science, UK. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. Chitosan (MW-25KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

2.2. Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines.

2.3. Preparation of GABA-Chitosan Nanoparticles and Morphological Characterization

The chitosan nanoparticles were prepared by ionic gelation method. Chitosan solution of 50 mL volume with concentration 1 mg/mL was prepared by dissolving chitosan in 2% acetic acid. The chitosan nanoparticles were precipitated from the solution by the addition of 33 mL of 1 mg/mL penta sodium tri polyphosphate (TPP) solution with vigorous stirring. To incorporate GABA in to chitosan, a solution of concentration 8.824 µg of GABA/mL of chitosan solution was prepared and the precipitation of GABA-chitosan nanoparticles were done by the above method. The precipitated nanoparticles were centrifuged at 16,000× g for 20 minutes. The pellet was washed with distilled water and then resuspended in saline. The SEM image of the nanoparticles was taken with a magnification of 20000× by scanning electron microscope (JEOL Model JSM-6390LV).

2.4. FT-IR Spectroscopy

The FT-IR spectrum of the GABA, chitosan, chitosan nanoparticles and GABA incorporated chitosan nanoparticles were taken using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) with spectral range of 4000–400 cm−1.

2.5. Determination of Encapsulation Efficiency and In Vitro Release of GABA

The maximum encapsulation efficiency of GABA with chitosan nanoparticles was obtained by giving emphasis to concentration of GABA added to the chitosan solution and the duration of reaction between GABA and chitosan. The encapsulation efficiency was calculated by incorporating [3H] GABA with chitosan and the radioactivity of the GABA, which was bound on the chitosan nanoparticles, were related to its concentration. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid...
Scintillation counter. % Encapsulation = (Concentration of GABA bound to chitosan nanoparticles/Concentration of GABA added initially) × 100. In in vitro release studies [3H] GABA-chitosan nanoparticles were suspended in PBS, pH 7.4 and stirred gently. At different intervals of time from 0 to 40 hours, the concentration of released radioactive GABA at each time from the nanoparticles was calculated to get a release profile in vitro.

2.6. Cell Uptake of GABA-Chitosan Nanoparticles

2.6.1. Preparation of FITC Labeled Chitosan Nanoparticles

Chitosan solution (1 mg/mL), of volume 50 mL was prepared and the nanoparticles were precipitated by adding TPP. The nanoparticles were centrifuged and the pellet was resuspended in 5 mL DMSO and sonicated for 1 minute. Then a solution of 10 mg/mL FITC in DMSO was added to the nanoparticle suspension. Stirred the solution gently and kept overnight at dark. After stirring, particles were washed with DMSO several times until the non conjugated FITC was eliminated completely. The liver was perfused initially with Ca2+ buffer, pH 7.4 (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES and 5.5 mM NaOH) and then with collagenase buffer, pH 7.6 (67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.76 mM CaCl2 · 2H2O, 0.66 mM NaOH and collagenase). The perfused liver was minced in PBS, pH 7.4 and kept for collagenase digestion. The cells were filtered and washed. Resuspended the cells in William’s media and 150 μL of cell suspension (cell density of 1.6 × 10^5 cells/cm²) was added to a four well glass slide. Then the cells were incubated in 5% CO₂ atmosphere for 24 hours at 37 °C.

2.6.2. Uptake of FITC Labeled Nanoparticles by Hepatocytes

50 μL of FITC labeled and unlabelled nanoparticles were added to the corresponding cell suspension in each well and incubated for 2 hours. After the incubation, the fluorescent images were captured using confocal microscope with an excitation at 488 nm.

2.6.3. Effect of GABA Encapsulated Chitosan Nanoparticles on DNA and Protein Synthesis in Hepatocytes

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16 hour fast. Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M to avoid diurnal variations in responses. After surgery, 1 mL of 30 μg/μL GABA-chitosan nanoparticles and 0.26 mg/mL pure GABA (same concentration of GABA in GABA-chitosan nanoparticles) suspended in saline were injected intra peritoneal to the respective rats. Sham operated control (C), partially hepatectomised group without any treatment (PHNT), partially hepatectomised group with pure GABA treatment (G) and partially hepatectomised groups with GABA-chitosan nanoparticle treatment (GCNP) were the four experimental groups. The rats were sacrificed by decapitation 24 hours post hepatectomy.

The liver from the sham operated control and remaining liver from all the other three groups were perfused and cultured for 24 hours. Before incubation [3H] thymidine of specific activity 18 Ci/mmol was added to one set of culture plates for all the four experimental groups to determine the measurement of DNA synthesis and [3H] thymidine (0.5 μCi), 10 mM ATP, 100 mM NaF, 10 mM MgCl2, 0.1 M Tris-HCl buffer, pH 8.0 and the liver supernatant fraction. After incubation at 37 °C for 15 minutes the reaction mixture was spotted in DE 81 paper discs. The bound radioactivity of [3H] thymidine in the presence of ATP to [3H] thymidine monophosphate (TMP) by the binding of latter nucleotide to DEAE cellulose discs. The reaction mixture contained 5 mM [3H] thymidine (0.5 g/mL), 10 mM ATP, 100 mM NaF, 10 mM MgCl2, 0.1 M Tris-HCl buffer, pH 8.0 and the liver supernatant fraction. After incubation at 37 °C for 15 minutes the reaction mixture was spotted in DE 81 paper discs. The bound radioactivity of [3H] thymidine monophosphate was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. DNA synthesis was further determined by analyzing the activity of thymidine kinase (TK) in all the four experimental groups. A 10% liver homogenate was prepared in 50 mM Tris HCl buffer, pH 7.5. It was centrifuged at 36000 × g for 30 minutes and the supernatant was collected. TK was assayed by determining the conversion of [3H] thymidine in the presence of ATP to [3H] thymidine monophosphate (TMP) by the binding of latter nucleotide to DEAE cellulose discs. The reaction mixture contained 5 mM [3H] thymidine (0.5 μ Ci), 10 mM ATP, 100 mM NaF, 10 mM MgCl2, 0.1 M Tris-HCl buffer, pH 8.0 and the liver supernatant fraction. After incubation at 37 °C for 15 minutes the reaction mixture was spotted in DE 81 paper discs. The bound radioactivity of [3H] thymidine monophosphate was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

2.6.4. Quantification of IP₃

The liver was homogenised in a poltron homogeniser in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 × g for 15 min. and the supernatant was transferred to fresh tubes for IP₃ assay using [3H] IP₃, Biotrak Assay System kit. The unknown concentrations were determined from the standard curve using appropriate dilutions and calculated for pmoles/mg protein. Protein was measured according to Lowry et al., using bovine serum albumin as standard. The intensity of the purple blue color formed was proportional to the amount of protein which was read in a spectrophotometer at 660 nm. A standard curve was plotted with % B/Bo on the Y-axis and IP₃.
of the baseline fluorescence. The intensity was 20-fold greater than the standard deviation.
of GABA and amino group of chitosan and thus a new shoulder peak at 1405.57 cm$^{-1}$ appeared. 1400.46 cm$^{-1}$ represented a weak COO$^-$ symmetrical peak and the influence of this was observed in GABA-chitosan nanoparticles at 1405.57 cm$^{-1}$ (salt of carboxyl) (Figs. 2(a–d)).

The efficiency of interaction of GABA with chitosan was studied by varying the time required for the interaction of GABA with chitosan and changing concentrations of GABA. A vigorous stirring of GABA with chitosan solution for 2 hours gave good encapsulation of 66 ± 7.1%. By changing the concentration of GABA from 0 to 35 μg and considering the chitosan solution of 1mg/mL, the maximum encapsulation of 93 ± 8.3% was obtained at a concentration 8.824 μg of GABA/mL chitosan solution (Figs. 3(a, b)). From in vitro release studies of GABA there was an initial burst release of 20% in 0.5 hours, 87% in 20 hours and 94% in 30 hours from the nanoparticles in PBS (Fig. 3(c)).
The chitosan-GABA nanoparticles were labeled with FITC by incubating the nanoparticles with FITC in DMSO overnight. The cultured hepatocytes were incubated in the presence of FITC labeled GABA chitosan nanoparticles and unlabelled nanoparticles for 2 hours. A negative control showing the auto fluorescence in the cell was obtained. The fluorescent image was observed in confocal microscope with an excitation at 488 nm. The interaction of FITC labeled nanoparticles with hepatocytes was observed. The binding of fluorescent labeled GABA-chitosan nanoparticles on the surface of hepatocytes and the entry of nanoparticles in to the hepatocytes by phagocytosis led to the observation of fluorescence in the cell (Figs. 4(a, b)).

Tritiated thymidine incorporation in to replicating DNA was used as a biochemical marker for quantifying DNA synthesis. After partial hepatectomy, 1 mL of 30 μg/μL concentration of GABA-chitosan nanoparticles suspended
Fig. 3. The maximum encapsulation efficiency by standardizing (a) reaction time for GABA and chitosan in hours, (b) concentration of GABA in μg/mL chitosan solution, and (c) in vitro release of [3H] GABA from nanoparticles in PBS.

in saline and 0.26 mg GABA in saline were injected intraperitoneally to groups GCNP and G respectively. After 24 hours the animals were sacrificed and the remaining liver from the groups PHNT, G and GCNP which was triggered for regeneration and non triggered liver from control were perfused and cultured for 24 hours in the presence of tritiated thymidine for quantifying DNA and tritiated leucine for quantifying protein. The uptake of tritiated thymidine and leucine were observed. The tritiated thymidine uptake in PHNT was significantly increased \((p < 0.01)\) with respect to sham operated control. There was also a significant increase in tritiated thymidine uptake by hepatocytes from group G when compared with control \((P < 0.01)\) and PHNT \((p < 0.05)\). In the GABA chitosan treated GCNP group a significant increase \((p < 0.001)\) with respect to control and \((p < 0.01)\) with respect to PHNT was observed. The tritiated thymidine uptake in GCNP showed a significant increase \((p < 0.01)\) with respect to G. For tritiated leucine uptake there was significant increase \((p < 0.05)\) in PHNT and G groups when compared with the sham operated control. There was

Fig. 4. The study of interaction between GABA-chitosan nanoparticles and hepatocytes in vitro using confocal microscope. (a) The bright field image (inset) and confocal image shows the interaction of non fluorescent GABA-chitosan nanoparticles with hepatocytes. (b) The bright field image (inset) and confocal image shows the interaction of FITC labeled GABA-chitosan nanoparticles with hepatocytes. The interaction of labeled nanoparticles at cell membrane and the engulfing of nanoparticles by hepatocytes lead to the fluorescence in the interior of the cell. The scale bars represent 15 μm.
compared to sham operated control (Table III).

In PHNT, G and GCNP are decreased \((p < 0.01)\) with respect to sham operated control. \(^{*}p < 0.01\) with respect to PHNT. \(^{**}p < 0.01\) with respect to G. Values are mean \(\pm\) S.E.M of 4-6 separate experiments.

### Table I. Tritiated thymidine uptake by hepatocytes obtained in control and experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>DPM/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>15897 ± 189</td>
</tr>
<tr>
<td>PHNT</td>
<td>16005 ± 543(^{a,b})</td>
</tr>
<tr>
<td>G</td>
<td>19865 ± 298(^{a,b})</td>
</tr>
<tr>
<td>GCNP</td>
<td>23358 ± 400(^{a,b})</td>
</tr>
</tbody>
</table>

C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA-chitosan nanoparticle treatment. \(^{*}p < 0.01\) with respect to sham operated control. \(^{*}p < 0.01\) with respect to PHNT. \(^{**}p < 0.01\) with respect to G. Values are mean \(\pm\) S.E.M of 4-6 separate experiments.

### Table II. Tritiated leucine uptake by hepatocytes obtained in control and experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>DPM/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>11218 ± 388</td>
</tr>
<tr>
<td>PHNT</td>
<td>12658 ± 392(^{a})</td>
</tr>
<tr>
<td>G</td>
<td>12986 ± 483(^{a})</td>
</tr>
<tr>
<td>GCNP</td>
<td>14416 ± 823(^{a})</td>
</tr>
</tbody>
</table>

C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment. \(^{*}p < 0.01\), \(^{**}p < 0.05\) with respect to sham operated control. \(^{***}p < 0.01\) with respect to PHNT. \(^{****}p < 0.05\) with respect to G. Values are mean \(\pm\) S.E.M of 4-6 separate experiments.

### Table III. Thymidine kinase assay in hepatocytes from control and experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vmax (nmoles/mg/min)</th>
<th>Km (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>17.30 ± 0.88</td>
<td>0.92 ± 0.03</td>
</tr>
<tr>
<td>PHNT</td>
<td>414.42 ± 82.20(^{a})</td>
<td>0.80 ± 0.02(^{c})</td>
</tr>
<tr>
<td>G</td>
<td>425.19 ± 95.90(^{a,b,c})</td>
<td>0.80 ± 0.03(^{c})</td>
</tr>
<tr>
<td>GCNP</td>
<td>479.84 ± 23.45(^{a,b,c})</td>
<td>0.80 ± 0.03(^{c})</td>
</tr>
</tbody>
</table>

C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment. \(^{*}p < 0.01\), \(^{**}p < 0.05\) with respect to sham operated control. \(^{***}p < 0.01\) with respect to PHNT. \(^{****}p < 0.01\) with respect to G. Values are mean \(\pm\) S.E.M of 4-6 separate experiments.

much more increase in the leucine uptake \((p < 0.01)\) of GCNP compared to both control and PHNT and significant increase \((p < 0.05)\) with respect to G (Tables I and II).

The enzyme thymidine kinase converts thymidine in to thymidine monophosphate, which is involved in the DNA synthesis. The thymidine kinase activity in all the four experimental groups showed a significant increase \((p < 0.001)\) in the activity of thymidine kinase comparing with PHNT, G and GCNP groups with sham operated control. Also, a significant increase \((p < 0.001)\) in thymidine kinase activity of liver from the groups G and GCNP with respect to PHNT was observed. A significant increase \((p < 0.001)\) in the enzyme activity for group GCNP when compared to G was obtained. The \(K_{m}\) value of the enzyme in PHNT, G and GCNP are decreased \((p < 0.05)\) when compared to sham operated control (Table III).

\[ IP_{3} \text{ content of the regenerating liver of PHNT, G and GCNP showed a significant decrease (} p < 0.001\) compared to sham operated control. The \( IP_{3}\) content of the liver of group G and GCNP was decreased with \( p < 0.01\) and \( p < 0.001\) respectively when compared to PHNT and for group GCNP the \( IP_{3}\) content showed a significant decrease \((p < 0.01)\) when compared to G (Fig. 5).

Gene expression studies of phospholipase C (PLC) in the regenerating liver of PHNT and G showed a significant decrease \((p < 0.01)\) and for GCNP with \( p < 0.001\) with respect to sham operated control. There was also a significant decrease \((p < 0.05)\) in PLC gene expression of the liver of G and GCNP when compared to PHNT. For group GCNP, there was a significant decrease \((p < 0.01)\) in the gene expression when compared to G (Fig. 6). The NF-κB expression in the regenerating liver of PHNT, G and GCNP showed a significant decrease \((p < 0.001)\) when compared to control and \( p < 0.001\) for G and GCNP with respect to PHNT and for GCNP when compared to G (Fig. 7).

Caspase-8 expression in the PHNT group was significantly
Fig. 7. Real time PCR amplification of NF-κB mRNA in the liver of experimental rats. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. *p < 0.001 with respect to control. †p < 0.001 with respect to PHNT. ‡p < 0.001 with respect to G. Values are mean ± S.E.M of 4–6 separate experiments. C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment.

Fig. 8. Real time PCR amplification of caspase-8 mRNA in the liver of experimental rats. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6-8 rats. *p < 0.01 and †p < 0.01 with respect to control. ‡p < 0.001 and §p < 0.05 with respect to PHNT. †p < 0.001 with respect to G. Values are mean ± S.E.M of 4–6 separate experiments. C—Sham operated control, PHNT—Partially hepatectomised group with no treatment, G—Partially hepatectomised group with GABA treatment and GCNP—Partially hepatectomised group with GABA-chitosan nanoparticle treatment.

decreased (p < 0.01) when compared to C. There was a significant decrease (p < 0.001) for G and GCNP with respect to C and a significant decrease (p < 0.05) for G and (p < 0.001) for GCNP when compared to PHNT. Also a significant decrease (p < 0.001) in Caspase-8 expression in GCNP when compared to G was also observed (Fig. 8).

4. DISCUSSION

Liver cell damage occurs in many ways. Alcohol-induced cell death and inflammation result in scarring that distorts the liver’s internal structure and impairs its function.28,29 Many drugs with analgesic and antipyretic action affect many peripheral tissues. However, an acute or cumulative over dose can cause severe liver injury with the potential to progress to liver failure. Acetaminophen is one such drug.30 Liver cell damage is also occurred due to the attack of parasites like Entamoeba histolytica.31 Apart from drugs, many hepatotoxic chemicals also cause liver cell apoptosis.32,33 Due to all these reasons the metabolic functions of one of the major organ in the body, liver, gets disturbed. So the proliferation of damaged hepatocytes is essential for the balanced routine body functions. Chitosan is a natural polymer produced by deacetylation of chitin. Chitin is the second abundant polymer in nature.34 It posses positive charge and thus interact with the negatively charged cell membrane. In the last two decades, Chitosan nanoparticles have been extensively explored for pharmaceutical applications35 like paclitaxel conjugated chitosan oligosaccharide for cancer therapy,36 encapsulation of nitrone in chitosan nanoparticles for the treatment of stroke.37 Gamma amino butyric acid (GABA), a neurotransmitter, enhances hepatocyte proliferation in vitro.13 Apart from the inhibitory role of GABA, it can also be used for the proliferation of damaged cells.

Spherical nanoparticles of chitosan bound to GABA were prepared and the morphological study was carried out with scanning electron microscope. The interaction of GABA with chitosan was confirmed by FT-IR study. A salt formation was observed during the interaction of GABA and chitosan. The maximum encapsulation efficiency of chitosan leads to better loading of GABA in chitosan nanoparticles. The mechanism of interaction of GABA and chitosan is the bond formation between the amino group of chitosan and carboxyl group of GABA (salt formation), which is clear from the FT-IR data. We observed maximum encapsulation efficiency by standardizing the concentration
of GABA and the reaction time for binding GABA with chitosan. The interaction of nanoparticles with the hepatocytes was observed with FITC-labelled nanoparticles under confocal microscope. The GABA-chitosan nanoparticles were administered through the peritoneal cavity for a better absorption of nanoparticles to partially hepatectomised liver. The chitosan nanoparticles are biocompatible and are degraded by the endosomal pathways in the cell and thus the therapeutic potential of chitosan polymer conjugates are being investigated for in vitro and in vivo applications. Gamma amino butyric acid is a non essential amino acid synthesized in the human body and also from the earlier reports confirmed the biocompatibility of GABA. After two third hepatectomy of liver, the major metabolic pathways are disrupted. The liver starts to regenerate and the maximum DNA synthesis occurs by 24 hours post hepatectomy. Thymidine is the nitrogen base, which is found only in DNA and the increase in DNA synthesis leads to enhanced utilization of thymidine. In proteins the major amino acid observed is leucine and thus an increase in leucine uptake by cells in vitro explains the enhancement in protein synthesis. DNA and protein syntheses were observed to be high in GCNP group compared with G and PHNT. This shows an increase in cell regeneration in the GABA-chitosan nanoparticle treatment groups. In the case of GABA chitosan nanoparticles, the less exposure of GABA to the internal body environment and the presence of positive charge and high cell binding affinity of chitosan the more effective receptor activation by GABA is achieved. The activity of liver thymidine kinase, an enzyme converts thymidine to thymidine monophosphate, is directly proportional to DNA thymidine kinase, an enzyme converts thymidine to thymidine monophosphate, is directly proportional to DNA synthesis. We observed the highest activity of thymidine kinase in GCNP group than G, PHNT and C.

After partial hepatectomy, an increase in apoptosis is also observed due to an increase in the level of reactive oxygen species (ROS) which in turn alter protein and nucleic acid structures. IP$_3$ receptor inactivation phenotypically mimics Bax deficiency by attenuating caspase-3 expression and activation. The IP$_3$ content in the liver of partially hepatectomised rats are reduced due to the triggering of cell division rather than apoptosis. Our results explain a further reduction in IP$_3$ content which is achieved by GABA-chitosan treatment and even decreased by the GABA-chitosan nanoparticle treatment. GABA through GABA$_A$ receptors which are G protein coupled receptors, influence many cell signaling pathways including the regulation of protein kinase C. The expression of Gi, alpha subunit of G protein is increased by 24 hours of hepatectomy and thus the activity of adenyl cyclase is also decreased. It was reported that there is a synergism between Phospholipase C and Adenyl cyclase linked growth factors. Phospholipase C catalyses the hydrolysis of phosphatidylinositol (4,5)-bisphosphate results in the production of diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP$_3$), which activate protein kinase C (PKC). So due to the reduction in adenyl cyclase the expression of phospholipase C also gets reduced which in turn reduces IP$_3$. The activators of GABA$_B$ receptors, significantly inhibited the accumulation of inositol-1-phosphate and inositol-1,4,5-trisphosphate in cells. Thus the reduction in both IP$_3$ content and the expression of phospholipase C support the fact of reduction in apoptosis. Our results emphasize an increase in cell proliferation by the reduction of IP$_3$ and phospholipase C. Also PLC related proteins activate GABA$_A$ receptors, which suppress the cell proliferation. Our results also showed reduction in PLC which leads to the suppression of GABA$_A$ receptors and cause enhancement of cell division.

The gene expression of NF-$\kappa$B was significantly reduced in the GABA treated and further decreased in GABA-chitosan treated partially hepatectomised rat liver when compared to PHNT and C. In PHNT also there was a significant reduction in NF-$\kappa$B than C. PKC activates transcription factors, such as NF-$\kappa$B, which leads to the regulation of the expression of genes involved in cell proliferation. The activated, NF-$\kappa$B is dissociated from its inhibitor, I-$\kappa$B, and translocated into the nuclei, where it induces transcriptional up regulation of various proinflammatory mediators such as TNF-$\alpha$. Even though TNF-$\alpha$ is an initial and mandatory cytokine for liver regeneration the suppression of TNF-$\alpha$ improves liver function and facilitate liver regeneration after extended hepaectectomy. Apoptosis could also be induced by death domain receptor ligands such as TNF-$\alpha$ and Fas ligand. So decreased NF-$\kappa$B expression leads to less activation of TNF-$\alpha$ and enhanced cell proliferation. Apoptosis after partial hepatectomy was further clarified by studying the gene expression of Caspase-8. Active Caspase-8 will further activate other Caspases like Caspase-3 and result in DNA fragmentation. From our study it is clear that the gene expression of Caspase-8 is decreased significantly in GCNP when compared to PHNT and G, which shows that the apoptosis is reduced in nanoparticle treated group.

5. CONCLUSION

The major after effect of liver disease is the destruction of active cells that play a vital role in the metabolism of many compounds in the body. So the regeneration of hepatocytes from damaged liver for the proper functioning of body gains immense interest. Treatment with GABA-chitosan nanoparticles in partially hepatectomised rat liver improves hepatic regeneration when compared to the regeneration due to pure GABA treatment and without any treatment. This has clinical significance in liver based diseases.

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Role of GABA and serotonin coupled chitosan nanoparticles in enhanced hepatocyte proliferation

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J. Shilpa · B. T. Roshni · R. Chinthu · C. S. Paulose

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Abstract The development of nanoparticles containing active molecules having improved stability, sustained release and maximum half life helps in cell proliferation result in enhanced tissue regeneration. Our study focuses on the use of Gamma amino butyric acid (GABA) and serotonin (5-HT) coupled chitosan nanoparticles for the active liver regeneration in male Wistar rats. The nanoparticles were prepared and the morphology was studied using SEM. The FT-IR spectra of the nanoparticles and the maximum encapsulation efficiency of GABA and 5-HT binding to chitosan nanoparticles were observed. The in vitro release studies provided the percentage release of GABA and 5-HT from the nanoparticles at different time intervals. The quantification of DNA and protein syntheses was done using $[^3]H$ thymidine and $[^3]H$ leucine uptake studies that determined the enhancement in hepatocyte proliferation. Our results project the role of GABA and 5-HT chitosan nanoparticles in the treatment of liver based diseases.

1 Introduction

Nanoparticulate drug delivery systems provide wide opportunities for solving problems associated with drug stability or disease states and create great expectations in the area of drug delivery [1]. Nanotechnology is the technology that deals with one billionth of a meter scale [2]. To a great extend, biopolymer coated drugs coupled with nanotechnology reduces the major drawbacks of the common drug delivery methods. Chitosan, deacetylated chitin which is a linear nitrogenous polysaccharide, is a copolymer of $\beta$-(1,4) linked glucosamine (deacetylated unit) and N-acetyl glucosamine (acetylated unit) [3]. Owing to the removal of acetyl moieties that are present in the amine functional groups of chitin, chitosan is readily soluble in aqueous acidic solution. The solubilisation occurs through the protonation of amino groups on the C-2 position of $\delta$-glucosamine residues whereby polysaccharide is converted into polycation in acidic media. Chitosan interacts with many active compounds due to the presence of amine group in it. The presence of this active amine group in chitosan was exploited for the interaction with the active molecules in the current study. Chitosan is nontoxic, biodegradable and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections [4–8]. Polymeric drug delivery systems can be used to deliver drugs directly to the intended site of action to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs [9].

A non proteinaceous amino acid Gamma amino butyric acid (GABA) is an important inhibitory neurotransmitter in the vertebrate central nervous system. Apart from the inhibitory role, GABA helps in the proliferation of cells in different parts of the body. The proliferative role of GABA was observed in the TM3 Leydig cell multiplication in testis [10], development of outer retina in rabbits [11] and promotes neurite growth, cell proliferation and migration [12]. A GABA agonist, baclofen, induced EGF mediated DNA synthesis in hepatocyte in vitro and an increase in hepatocyte proliferation was observed through the activation of GABA$_B$ receptors. Also, it significantly reduced the TGF $\beta$1 suppression of EGF induced DNA synthesis.
Thus the activation of GABA receptors triggers DNA synthesis, which is mediated through the G protein, in primary cultures of rat hepatocytes [13]. The expression of the inhibitory and stimulatory x-subunit of G proteins coupled receptors to the targets like adenylate cyclase cause biphasic increase in hepatic cAMP and thus an enhanced signalling for cell division.

Serotonin (5-HT) has been shown to be mitogenic in many cells other than neuronal, exerting its effect by its receptor-mediated second messenger pathways. One of the factor influences the rate of dentate gyrus neurogenesis is 5-HT [14]. The S2 receptor subtype of 5-HT has been shown to have mediated cell growth in fibroblasts [15]. The 5-HT S2 receptor has been cloned in the human liver and has been shown to have a high degree of homology with the S2 receptors of rat and mouse liver [16]. The S2 receptors of 5-HT are coupled to phospho-inositol turnover and diacylglycerol formation, which activates protein kinase C (PKC), an important second messenger for cell division [17]. 5-HT S2 receptor–mediated activation of PKC has been shown to result in the phosphorylation of a 40 kDa substrate protein of PKC in human platelets [18]. Platelets are major carriers of 5-HT in the blood. It was reported that 5-HT can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes [19].

In the present study, the synthesis of nanoparticles by GABA and 5-HT with chitosan and their delivery for active liver regeneration was emphasised. The liver regeneration in partially hepatectomised rats has contributed a powerful model for evaluating the hepatocyte proliferation in vivo [20]. The liver is an important body organ responsible for various functions like bile production, storage and filtering of blood, metabolism of fats and sugars and thus making compounds which control blood volumes and clotting. Liver cell damage is occurred due to several reasons. Liver’s internal structure is distorted due to the scars formed as a result of alcohol induced cell death and inflammation [21, 22]. One in twenty-five deaths World-wide are directly related to alcohol consumption. An over dose of many drugs cause severe liver injury which leads to liver failure. Liver cell destruction is also observed due to the attack of parasites like Entamoeba histolytica [23]. Many hepatotoxic chemicals like dyes, preservatives and insecticides cause liver cell apoptosis [24, 25]. The regeneration of damaged liver is considered as a solution for all disturbing factors of normal hepatocytes’ functions. A two-third partial hepatectomy (PH) of rats induces the remainder of the liver to undergo a synchronous first wave of DNA synthesis, followed by several rounds of mitosis [26–29]. Mitosis begins 6 h later and peaks at 30 h. The original DNA content of the regenerating liver remnant is normally restored within 96 h after PH [30].

The present work elucidates the role of GABA and 5-HT coupled chitosan nanoparticles triggering the hepatocyte proliferation in partially hepatectomised male Wistar rats, in vivo. The standardisation of nanoparticle synthesis was done to achieve a maximum binding of GABA and 5-HT with chitosan. An enhanced cell proliferation in the nanoparticle treated groups was observed by quantifying DNA and protein syntheses.

2 Materials and methods

2.1 Chemicals used and their sources

Biochemicals, including GABA (mw, 103.12) and 5-HT (mw, 387.41), were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally, [3H]GABA (Sp. Activity 76.2 Ci mmol⁻¹), [3H] thymidine (Sp. Activity 18.0 Ci mmol⁻¹) and [3H] leucine (Sp. Activity 63.0 Ci mmol⁻¹) were purchased from Amersham Life Science, UK. Chitosan (mw, 25 kDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

2.2 Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 h light and 12 h dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats were divided into four experimental groups: sham operated control (C), partially hepatectomised rats with no treatment (PHNT), partially hepatectomised rats with 5-HT chitosan nanoparticle treatment (SCNP) and partially hepatectomised rats with a combination of GABA and 5-HT chitosan nanoparticle treatment (GSCNP). Each group consisted of 6–8 animals.

2.3 Preparation of neurotransmitter-chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method [31]. Chitosan was dissolved in 2 % acetic acid to get chitosan solution of concentration 1 mg mL⁻¹. Chitosan nanoparticles from 50 mL chitosan solution were precipitated by the addition of 33 mL of 1 mg mL⁻¹ penta sodium tri polyphosphate (TPP) solution with rapid stirring. To incorporate 5-HT creatinine sulphate to chitosan,
300 μg of 5-HT mL\(^{-1}\) of chitosan solution was prepared and the precipitation of 5-HT chitosan nanoparticles were done by the above method. To prepare a combination of GABA and 5-HT chitosan nanoparticles, 400 μg of 5-HT and 20 μg of GABA were dissolved in chitosan solution and the nanoparticles were precipitated by the addition of TPP. The precipitated nanoparticles were centrifuged for 20 min at 16,000×g. The pellet was washed thoroughly with distilled water and then resuspended in saline. The SEM image of the nanoparticles was taken with a magnification of 10,000× by scanning electron microscope (JEOL Model JSM–6390LV).

2.4 FT-IR spectroscopy

The FT-IR spectrum of the 5-HT, 5-HT incorporated chitosan nanoparticles and GABA and 5-HT incorporated chitosan nanoparticles were taken using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) with a spectral range of 4,000–400 cm\(^{-1}\).

2.5 Determination of encapsulation efficiency and in vitro release studies

The maximum encapsulation efficiency [32] of 5-HT with 5-HT chitosan nanoparticles and GABA and 5-HT with GABA and 5-HT chitosan nanoparticles was obtained by giving emphasis to concentration of GABA or 5-HT added to the chitosan solution. The encapsulation efficiency of GABA in GABA and 5-HT chitosan nanoparticles was calculated by incorporating \(^{3}H\) GABA with chitosan and the radioactivity of the GABA, which was bound on the chitosan nanoparticles, were related to its concentration [33, 34]. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

The concentration of 5-HT, which was bound to the nanoparticles, were found by HPLC with electrochemical detector (Waters, USA) fitted with CLS-ODS reverse phase column of 5 μm particle size. After centrifugation of nanoparticle suspension, the supernatant with unbound 5-HT was filtered through 0.22 μm HPLC grade filters and injected to the column. The mobile phase consisted of 50 mM sodium phosphate dibasic, 0.03 M citric acid, 0.1 mM EDTA, 0.6 mM sodium octylsulfonate and 15 % methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 μm (Millipore) and degassed. A Waters model 515, Milford, USA, pump was used to deliver the solvent at a rate of 1 mL min\(^{-1}\). The 5-HT was identified by amperometric detection using an electrochemical detector (Waters model 2465) with a reduction potential of +0.80 V. The peaks obtained were compared with standard creatinine sulphate and quantitatively estimated using an integrator (Empower software) interfaced detector.

% Encapsulation =

\[
\frac{\text{Concentration of GABA or 5-HT bound to chitosan nanoparticles/concentration of GABA or 5-HT added initially}}{\times 100}
\]

In in vitro release studies, \(^{3}H\) GABA and 5-HT chitosan nanoparticles and 5-HT chitosan nanoparticles were suspended in PBS, pH 7.4. Both were gently stirred at different time intervals from 0 to 40 h. The concentration of released radioactive GABA [33] and 5-HT at each time from the nanoparticles was calculated to get a release profile in vitro.

2.6 Effect of GABA and 5-HT encapsulated chitosan nanoparticles on DNA and protein syntheses in hepatocytes

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anaesthesia, following a 16 h fast [26]. Sham operations involved median excision of the body wall followed by all manipulations except removal of lobes. All rats were undergone surgeries between 7 and 9 a.m. to avoid diurnal variations in responses. After surgery, 1 mL of 30 μg μL\(^{-1}\) 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats. The DNA synthesis prior to first mitotic phase occurs between 20 and 24 h after PH [28]. So the quantification of DNA and protein syntheses performed during this period provided a significant comparison among the experimental groups. Thus the rats were sacrificed by decapitation 24 h post hepatectomy.

The liver from the group C and remaining liver from all the other three groups were perfused and cultured for 24 h [34]. Cell suspension of 150 μL (cell density of 1.6 × 10\(^{5}\) cells cm\(^{-2}\)) was added to a four well poly L-lysine coated glass slide. Then the cells were incubated for 24 h at 37 °C in 5 % CO\(_2\) atmosphere. Before incubation, \(^{3}H\) leucine of specific activity 63 Ci mmol\(^{-1}\) to one set of culture plates for all the four experimental groups to determine the protein synthesis and \(^{3}H\) thymidine of specific activity 18 Ci mmol\(^{-1}\) to determine the measurement of DNA synthesis. All the experiments were done in triplicates. The cells were scrapped off from the culture plates and centrifuged at 2,000×g for 20 min. The supernatant was discarded and the pellet was resuspended in 50 μL, 1 M NaOH and kept overnight. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

2.7 Statistics

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a,
Student–Newman–Keuls test was used to compare different groups after ANOVA. ANOVA assumes that all the data follow Gaussian distribution and the tests were done by the method of Kolmogorov and smirnov.

3 Results

5-HT chitosan and GABA and 5-HT chitosan nanoparticles were prepared by the method of ionic gelation. The morphology of nanoparticles was observed by SEM (Fig. 1a, b). FT-IR spectra of 5-HT, 5-HT chitosan nanoparticles and GABA and 5-HT in combination with chitosan nanoparticles were studied. –NH stretch at 3,436 cm\(^{-1}\) in chitosan nanoparticle was changed to broadened peaks from 3,464.72 to 3,280 cm\(^{-1}\) on binding with 5-HT. There was a shift in C–N stretch of amine in 5-HT when the 5-HT chitosan nanoparticles were formed. In 5-HT chitosan nanoparticles, the S–O bond at 1,080.79 cm\(^{-1}\) and a short peak at 1,230.44 cm\(^{-1}\) was seen. In the nanoparticle, a new peak at 1,386.87 cm\(^{-1}\) was formed which denotes the S–N interaction due to the reaction between sulphate ion in 5-HT and NH\(^{+}\) ion in the chitosan. In GABA and 5-HT combination with chitosan nanoparticles, new and modified peaks were observed due to the presence of GABA and 5-HT. In pure GABA, –CH aliphatic stretch at 2,957 and 2,920 cm\(^{-1}\), which were overlapping with –NH stretching bands changed to 2,924.8 and 2,810 cm\(^{-1}\) due to the interaction with chitosan. The peak at 1,384.99 in chitosan nanoparticle, is a shoulder peak in the nanoparticles with GABA and 5-HT due to the formation of salt between GABA and chitosan. A peak at 1,072.44 cm\(^{-1}\), which denotes the S–O bond in the nanoparticles, shows the presence of 5-HT. A weak asymmetric –NH binding at 1,642 cm\(^{-1}\) in GABA became broad in GABA and 5-HT chitosan nanoparticles (Fig. 2a–c).

In 5-HT chitosan nanoparticles, changing the concentration of 5-HT from 0 to 400 µg mL\(^{-1}\) of chitosan solution (1 mg mL\(^{-1}\)), a maximum encapsulation of 86 ± 7.1 % was obtained at a concentration 300 µg of 5-HT mL\(^{-1}\) chitosan solution. Considering the encapsulation of 5-HT in GABA and 5-HT chitosan nanoparticles for a concentration range of 0–35 µg GABA mL\(^{-1}\) of chitosan solution (Fig. 3a–c). From the in vitro release studies of 5-HT, there was an initial burst release of 28 ± 2 % in 0.5 h and 96 ± 8.6 % in 30 h from the 5-HT nanoparticles in PBS. An initial 5-HT release of 30 ± 2 % in 0.5 h was observed in GABA and 5-HT chitosan nanoparticles and the release continued up to 97 ± 8.9 % at 20 h. From the GABA and 5-HT chitosan nanoparticles, there was an initial burst release of 22 ± 2 % GABA by 0.5 h and 95 ± 8.6 % by 30 h (Fig. 4a, b).

Incorporation of \(^{3}\text{H}\) thymidine into the replicating DNA was used as a biochemical marker to quantify DNA synthesis. The \(^{3}\text{H}\) thymidine uptake for PHNT was significantly increased \((p < 0.01)\) when compared to C. There was a significant increase \((p < 0.001)\) in \(^{3}\text{H}\) thymidine uptake by hepatocytes of SCNP when compared with C.
Fig. 2 **FT-IR spectrum** with a spectral range of 4,000–400 cm\(^{-1}\), using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) of a serotonin creatinine sulphate (5-HT), b 5-HT chitosan nanoparticles, and c GABA and 5-HT chitosan nanoparticles.
and PHNT ($p < 0.05$). In GSCNP group, a significant increase ($p < 0.001$) in $[^3]$H thymidine uptake when compared to C and PHNT was observed. For $[^3]$H leucine uptake studies, there was a significant increase ($p < 0.05$) in PHNT when compared with C. There was a significant increase ($p < 0.01$) in the leucine uptake by hepatocytes in SCNP when compared to C and PHNT. While considering the GSCNP group, there was also a significant increase ($p < 0.05$) in $[^3]$H leucine uptake when compared to PHNT (Tables 1, 2).

4 Discussion

Chitosan is the second-most abundant natural polysaccharide next to cellulose. Among other drug delivery strategies, a great deal of focus has been directed to chitosan nanoparticles to improve drug bioavailability, modify pharmacokinetics and/or protect the encapsulated drug [35]. Chitosan nanoparticles are prepared by the interaction of oppositely charged compounds. TPP has often been used to prepare chitosan nanoparticles because TPP is multivalent, nontoxic and able to form gels through ionic interactions. The interaction can be controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution [36]. Fewer side effects, solubility, poor bioavailability, specific
Healthy hepatocytes are necessary for the routine metabolic processes in the body. So active hepatocyte proliferation is important to reconstitute body functions after PH in rats. Earlier studies showed that GABA [13] and 5-HT [19] helped in the proliferation of hepatocytes in vitro. Our study focuses on the preparation of GABA and 5-HT combined chitosan nanoparticles for the hepatocyte proliferation in vivo. The morphology of the nanoparticles was visualized through scanning electron microscope. The interaction between GABA and 5-HT with chitosan was studied by FT-IR spectroscopy.

The maximum encapsulation efficiency of active compound to chitosan results in better loading of GABA and 5-HT in chitosan nanoparticles. From our earlier studies [34], the mechanism of interaction between GABA and chitosan is the bond formation between the amino group of chitosan and carboxyl group of GABA (salt formation). During the interaction of 5-HT and chitosan, a bond between sulphate ion in 5-HT creatinine sulphate and NH$_3^+$ ion in the chitosan is formed, which is clear from the FT-IR data. From the study, a maximum encapsulation efficiency of the active compounds with chitosan nanoparticles was observed by standardizing the concentration of GABA and 5-HT reacting with chitosan. The in vitro release studies showed the pattern of release of GABA and 5-HT from GABA and 5-HT chitosan nanoparticles and also 5-HT from 5-HT chitosan nanoparticles.

The nanoparticles were administered through the peritoneal cavity for achieving a better absorption of nanoparticles to partially hepatectomised liver [39]. The chitosan nanoparticles are biocompatible and are degraded by the endosomal pathways in the cell and thus the therapeutic potential of chitosan polymer conjugates are being investigated for in vitro and in vivo applications [40]. GABA is a non essential amino acid synthesized in the human body and also earlier reports confirmed the bio-compatibility of GABA [41]. Biju et al. [13] reported that GABA receptor activation occurs during liver regeneration.

5-HT receptor expression is increased after PH and it was reported that blockage of S$_2$ receptors arrested liver regeneration [42]. In thrombocytopenic mice, a 5-HT agonist reconstituted liver proliferation. The expression of 5-HT$_{2A}$ and 5-HT$_{2B}$ subtype 5-HT receptors in the liver increased after hepatectomy [43]. Thus activation of GABA and 5-HT receptor expression by the corresponding ligands achieved an enhancement in liver regeneration. Major metabolic pathways are disrupted after two-third hepatectomy of liver. During liver regeneration the maximum DNA synthesis occurs by 24 h post hepatectomy [44].

Thymidine is a nitrogen base that is found only in DNA and the increase in DNA synthesis results in enhanced utilization of thymidine. In proteins, the major amino acid observed is leucine and thus an increase in leucine uptake by cells explains the enhancement in protein synthesis. The DNA and protein synthesis quantitative studies for GABA

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>DPM (mg protein)</th>
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<tbody>
<tr>
<td>PHNT</td>
<td>18012 ± 540$^b$</td>
</tr>
<tr>
<td>SCNP</td>
<td>20876 ± 302$^{a,c}$</td>
</tr>
<tr>
<td>GSCNP</td>
<td>25969 ± 300$^{a,d}$</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 4–6 separate experiments

C Sham operated control, PHNT Partially hepatectomised group with no treatment, SCNP Partially hepatectomised group with 5-HT chitosan nanoparticle treatment, GSCNP Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

$^a$ $p < 0.001$ with respect to sham operated control

$^b$ $p < 0.01$ with respect to sham operated control

$^c$ $p < 0.05$ with respect to PHNT

$^d$ $p < 0.001$ with respect to PHNT

### Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>DPM (mg protein)</th>
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<tbody>
<tr>
<td>C</td>
<td>11226 ± 380</td>
</tr>
<tr>
<td>PHNT</td>
<td>12663 ± 397$^a$</td>
</tr>
<tr>
<td>SCNP</td>
<td>14534 ± 421$^{b,c}$</td>
</tr>
<tr>
<td>GSCNP</td>
<td>15984 ± 835$^{c,d}$</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 4–6 separate experiments

C Sham operated control, PHNT Partially hepatectomised group with no treatment, SCNP Partially hepatectomised group with 5-HT chitosan nanoparticle treatment, GSCNP Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment

$^a$ $p < 0.05$ with respect to sham operated control

$^b$ $p < 0.01$ with respect to sham operated control

$^c$ $p < 0.01$ with respect to PHNT

$^d$ $p < 0.001$ with respect to sham operated control

$^e$ $p < 0.001$ with respect to PHNT
chitosan nanoparticle treated group were carried out in our early studies [34]. Fasting animals prior to operation would probably not have diminished the variations in mitotic activity [45]. DNA and protein syntheses were observed to be high in GSCNP when compared to other groups. This shows an increased cell proliferation in the combined GABA and 5-HT chitosan nanoparticle treated group than the individual treatment with GABA or 5-HT coupled chitosan nanoparticles. In the case of GSCNP and SCNP groups, there is less exposure of GABA and 5-HT to the internal body environment and the presence of positive charge and high cell binding affinity of chitosan [46], more effective receptor activation by GABA and 5-HT were achieved.

Active liver cell proliferation is a value added process in medical field. Liver regeneration is a complex and multifactorial process that is regulated by various cell signalling cascades including the interactions between growth factors, regenerative cytokines and metabolic demand of the liver following surgery [47]. The active compounds that mediate the cell signalling mechanism for an enhanced DNA and protein formation prior to cell division, will promote an efficient and fast proliferation of cells. Our study discussed the involvement of neurotransmitters combination such as GABA and 5-HT, which are coupled to chitosan nanoparticles, in the synthesis of DNA and protein during the active cell multiplication in partially hepatectomised rat liver. The combined effect of improved stability and action of GABA and 5-HT on regenerating hepatocytes gives valuable scope for further studies in neurotransmitter involved liver regeneration.

5 Conclusion

Liver is an important organ that controls major metabolic functions in the body. Damage to liver even causes mood fluctuations and behavioural alterations. The major result of liver disease is the destruction of active hepatocytes that regulate the vital metabolism of many compounds in the body. So the regeneration of hepatocytes from damaged liver for the proper functioning of body gains immense scope. Treatment with GABA and 5-HT chitosan nanoparticles in partially hepatectomised rat liver improves hepatic regeneration when compared to the natural regeneration without any treatment. This has important clinical significance in liver related diseases.

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Increased Neuronal Survival in the Brainstem During Liver Injury: Role of γ-Aminobutyric Acid and Serotonin Chitosan Nanoparticles

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γ-Aminobutyric acid (GABA) and serotonin (5-HT)-mediated cell signaling, neuronal survival enhancement, and reduced neuronal death in brainstem during liver injury followed by active liver regeneration have a critical role in maintaining routine bodily functions. In the present study, GABAβ and 5-HT2A receptor functional regulation, interrelated actions of neuronal survival factors, and expression of apoptotic factors in the brainstem during GABA and 5-HT chitosan nanoparticles-induced active liver regeneration in partially hepatectomized rats were evaluated. Partially hepatectomized rats were treated with the nanoparticles, and receptor assays and confocal microscopic studies of GABAβ and 5-HT2A receptors, gene expression studies of GABAβ and 5-HT2A receptors, nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), Akt-1, phospholipase C, Bax, and caspase-8 were performed with the brainstems of experimental animals. A significant decrease in GABAβ and 5-HT2A receptor numbers and gene expressions denoted a homeostatic adjustment by the brain to trigger the sympathetic innervations during elevated DNA synthesis in the liver. The neuronal apoptosis resulting from the loss of liver function after partial hepatectomy was minimized by nanoparticle treatment in rats compared with rats with no treatment during regeneration. The present study revealed the potential of GABA and 5-HT chitosan nanoparticles for increasing neuronal survival in the brainstem during liver injury following regeneration, which avoids many neuropsychiatric problems.

The brain plays an important regulatory role in hepatic functions (O’Grady, 2005). The liver is a vital organ involved in routine metabolism in the body. Liver injury-related brain damage and death most frequently result from brainstem herniation resulting from increased intracranial pressure or brain edema resulting from altered ammonia and aromatic amino acid metabolism (Blei, 1991). This also affects various neurotransmitters and their receptor activation in brain. Deficiency of thiamine leads to brainstem neuronal loss (Cogan et al., 1985). Thus metabolism of the body with an injured liver is disturbed, which leads to several neuropsychiatric and mood alterations.

The regeneration of damaged liver automatically restores brain functions. Liver damage occurs due to overconsumption of alcohol, drugs with analgesic and antipyretic action, attack of parasites, and hepatotoxic chemicals. The presence of various comitogens is necessary for the successful and complete restoration of hepatic mass (Riehle et al., 2011). Prolonged liver dysfunction causes hepatocyte damage and fatal brain disorders. Several reports highlight liver injury-related brain damage (Cordoba and Blei, 1995; Butterworth, 1998; Chung et al., 2001; Larsen and Wendon, 2002), but activated liver cell proliferation coupled with increased neuronal survival has not been not well studied. The reduction in apoptosis enhances the survival of cells. The signals evoked by inositol (1,4,5)-triphosphate (IP3)- and phospholipase C-mediated Ca2+ ions in mitochondria trigger apoptosis. This was further studied from the expression patterns of caspase-8 and Bcl-2–associated X protein (Bax). Inflammatory mediators such as tumor necrosis factor-α (TNF-α) and neuronal survival factors such as nuclear factor-κB (NF-κB) and Akt-1 also play an important role in the regulation of brain function by the liver (Larsen and Wendon, 2002).

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γ-Aminobutyric acid (GABA) type B and serotonin (5-HT) type 2A receptors belong to the super family of G-protein–coupled receptors. GABA receptors are widely distributed in mammalian brain. Brain GABAergic changes are reported to regulate autonomic nerve function in rats (Martin and Haywood, 1998). In animal models of liver injury, an increase in GABAergic tone has been demonstrated (Albrecht and Jones, 1999). 5-HT is another neurotransmitter. Neurons participate in the regulation of sympathetic nerve discharge and have an inhibitory influence on central sympathetic pathways. 5-HT regulates cell proliferation, migration, and maturation in a variety of cell types. 5-HT has been implicated more in behavior, physiological mechanisms, and disease processes than any other brain neurotransmitter.

Nanoparticulate drug delivery systems provide immense hope for solving problems associated with current drug delivery methods. Chitosan, deacetylated chitin, is a copolymer of β-(1,4)-linked glucosamine (deacetylated unit) and N-acetyl glucosamine (acetylated unit; Radhakumary et al., 2005). Chitosan is a biodegradable, nontoxic, and biocompatible polymer. Chitosan nanoparticulate delivery systems are used to deliver drugs directly to the intended site of action to improve pharmacological efficiency by minimizing undesired side effects elsewhere in the body and decrease the long-term use of many drugs. Bioactive molecules are successfully encapsulated to improve bioavailability and bioactivity and to control delivery. The administration of pure neurotransmitters to the body results in degradation of the molecules and also vigorous binding to their receptors, which leads to receptor masking and cell signal arrest. Chitosan is inert to the internal body environment and thus the adverse side effects resulting from the interaction of neurotransmitters to organs other than the target organ are avoided. Chitosan encapsulation favors a controlled release than the rapid influx of active compounds.

We previously studied the role of GABA and 5-HT chitosan nanoparticles treatment individually and in combination in partially hepatectomized rats for enhanced hepatocyte proliferation (Shilpa et al., 2012a,b). Brainstem neurons are involved in the cardiovascular and respiratory control, alertness, and consciousness. Thus, brainstem damage is often a life-threatening problem. The present study assessed the potential of GABA and 5-HT chitosan nanoparticle treatment to reduce brainstem neuronal damage in partially hepatectomized rats. For achieving the aim, GABA_B and 5-HT_2A receptor functional regulation, gene expression of neuronal survival, and apoptotic factors in the brainstem during GABA and 5-HT chitosan nanoparticles-induced active liver regeneration in rats were studied.

**MATERIALS AND METHODS**

**Chemicals Used and Their Sources**

Biochemicals and the Tri reagent kit were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade and were purchased locally. [3H]baclofen (specific activity 42.9 Ci/mmol) and [3H]ketanserin (specific activity 63.3 Ci/mmol) were purchased from Amersham Life Science (Amersham, United Kingdom). Chitosan (MW 25 kDa) was a gift from the Central Institute of Fisheries Technology, Cochin, India.

**Animals**

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12-hr-light and 12-hr–dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were in accordance with the institutional, National Institutes of Health, and CPCSEA guidelines. All efforts were made to minimize animal suffering. Each group consisted of five animals. Sham-operated control (C), partially hepatectomized group without any treatment (PHNT), partially hepatectomized group treated with GABA chitosan nanoparticle (GCNP), partially hepatectomized group treated with 5-HT chitosan nanoparticle (SCNP), and partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle (GSCNP) were the five experimental groups.

**Preparation of GABA and 5-HT Chitosan Nanoparticles**

The chitosan nanoparticles were prepared by the ionic gelation method (Calvo et al., 1997). The incorporation of GABA and 5-HT into chitosan nanoparticles individually and in combination, standardization of encapsulation efficiency, and in vitro release profile studies were performed according to Shilpa et al. (2012a,b). The nanoparticles were washed thoroughly and were dispersed in saline.

**Partial Hepatectomy and Sacrifice**

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16-hr fast (Higgins and Anderson, 1931). Sham operations involved median excision of the body wall, followed by all manipulations except removal of the lobes. All the surgeries were performed between 7 and 9 AM to avoid diurnal variations in responses. After surgery, 1 ml of 30 µg/µl GABA chitosan nanoparticles, 5-HT chitosan nanoparticles, and a combination of GABA and 5-HT chitosan nanoparticles suspended in saline were injected intraperitoneally into the rats. The rats were sacrificed by decapitation 24 hr posthepatectomy, and brain and brainstem was dissected out quickly and kept over ice according to the procedure of Glowinski and Iversen (1966). The tissues were stored at −80°C until assayed.

**GABA_B and 5-HT_2A Receptor Binding Studies in the Brainstem Using [3H]Baclofen and [3H]Ketanserin**

[3H]Baclofen binding to GABA_B receptor in the membrane preparations was assayed (Hills et al., 1987). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.4, containing 2 mM CaCl_2 and 0.3–0.4 mg protein. In saturation binding experiments, 10–100 nM [3H]baclofen was incubated with and without excess of 100 µM unlabeled baclofen. The incubations were carried out at 20°C for 20 min. The
binding reactions were terminated by centrifugation at 14,000g for 10 min. The dried pellet was resuspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

$[^{1}H]$ketanserin binding to 5-HT$_{2A}$ receptor in the crude synaptic membrane preparation was carried out according to the modified procedure of Lysen et al. (1982). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.6, containing 0.3–0.4 mg protein. In saturation binding experiments, assays used different concentrations of 0.5–10 nM $[^{1}H]$ketanserin, which was incubated with and without excess of unlabeled 10 μM ketanserin. Tubes were incubated at 37°C for 15 min and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml ice-cold 50 mM Tris sulfate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The receptor binding parameters were determined via Scatchard analysis (Scatchard, 1949). The specific binding was determined by subtracting nonspecific binding from the total binding. The binding parameters maximal binding ($B_{\text{max}}$) and equilibrium dissociation constant ($K_d$) were derived by linear regression analysis by plotting the specific binding of the radioligand on the x-axis and bound/free on the y-axis. The maximal binding is a measure of the total number of receptors present in the tissue, and the equilibrium dissociation constant is a measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

Analysis of Gene Expression by Real-Time Polymerase Chain Reaction

PCR analyses were conducted with gene-specific primers and fluorescently labeled Taqman probe of GABA$_B$, 5-HT$_{2A}$, phospholipase C, NF-κB, TNF-α, Akt-1, Bax, and caspase-8, which were designed by Applied Biosystems (Foster City, CA). The endogenous control, β-actin, was labeled with a report dye, VIC. RNA was isolated from the brainstem of experimental rats using the Tri reagent according to the procedure of Chomczynski and Sacchi (1987). Total cDNA synthesis was performed with an ABI Prism cDNA archive kit in 0.2-ml microfuge tubes. The reaction mixture of 20 ng total RNA, 10× RT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/μl), and RNase-free water. The cDNA synthesis reactions were carried out at 25°C for 10 min and 37°C for 2 hr using an Eppendorf Personal Cycler. Real-time PCR assays were performed in 96-well plates in an ABI 7300 real-time PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems. The TaqMan reaction of 20 μl contained 25 ng total RNA-derived cDNAs; 200 nM each of the forward primer, reverse primer, and TaqMan probe for assay on demand; endogenous control β-actin; and 12.5 μl TaqMan 2× Universal PCR Master Mix (Applied Biosystems), and the volume was made up with RNase-free water. The following thermal cycling profile was used (40 cycles), 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCt method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (Ct) values of the target mRNAs to the Ct values of the internal control β-actin in the same samples (ΔCt = Ct target − Ctβ-actin). It was further normalized with the control (ΔΔCt = ΔCt − Ct control). The fold change in expression was then obtained as $2^{-\Delta\Delta Ct}$.

Immunohistochemical Analysis by Confocal Microscopy

The experimental rats were deeply anesthetized and transecially perfused with PBS (pH 7.4), followed by 4% paraformaldehyde in PBS (Chen et al., 2007). After perfusion, the brainstem from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in PBS (0.1 M). Ten-micrometer brainstem sections were cut with a cryostat (Leica CM1510 S). The sections were washed with PBS and then blocked for 1 hr with PBS containing 5% normal goat serum and 0.1% Triton X-100. The primary antibodies of GABA$_B_1$ (1:500 dilution in PBS with 5% normal goat serum and 0.1% Triton X-100) and 5-HT$_{2A}$ (1:1,000 dilution in PBS with 5% normal goat serum and 0.1% Triton X-100) were added to the various sections and incubated overnight at 4°C. After overnight incubation, the brain slices were rinsed with PBS and then incubated with fluorescence-labeled secondary antibody (Alexa Fluor 594; code A11012) prepared in PBS with 5% normal goat serum and 0.1% Triton X-100. The sections were washed with PBS thoroughly and then observed and photographed using a confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure was validated by negative controls (data not shown) to ensure that the labeling method accurately identified the antibody bound to the specific receptors in the brainstem. Expression of GABA$_B_1$ and 5-HT$_{2A}$ receptors was analyzed by using the pixel intensity method. The given pixel value is the net value deducted from the negative control pixel value (Joseph et al., 2010). Quantification was in the Leica application suit advanced fluorescence (LASAF) software, considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system, such as PMT, pinhole, and zoom factor, were kept same for imaging the sections of all experimental groups.

Narrow Beam Walk Test

The motor deficit was studied by using a narrow beam walk test. After 3 days posthepatectomy and treatment, the animals were tested for balance and motor coordination on a narrow beam maze (Allbutt and Henderson, 2007). This has a smooth, wooden, narrow beam 105 cm long, 4 cm wide, and 3 cm thick. The beam was elevated above the ground by 1 m with additional supports. It has a start platform of 20 cm in dimension from the
start of the beam and an end platform of 20 cm dimension at the end of 105-cm-long beam. There was food on the end platform as reward for the animals. The journey time between start and end was measured. The time was recorded when the animal placed a weight-bearing step entirely over the start line. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min.

Statistical Analysis

Statistical evaluations used analysis of variance (ANOVA) in GraphPad Instat (version 2.04a; GraphPad, San Diego, CA). Student Newman-Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made in Sigma Plot (version 2.03). Relative Quantification software was used for analyzing real-time PCR results.

RESULTS

GABA\textsubscript{B} and 5-HT\textsubscript{2A} Receptor Analysis in the Brainstem of Experimental Rats

B\textsubscript{max} represents the number of receptors, and K\textsubscript{d} denotes the affinity of receptors toward the ligand. B\textsubscript{max} of GABA\textsubscript{B} and 5-HT\textsubscript{2A} receptor binding studies showed a significant decrease (P<0.001) in PHNT, GCNP, SCNP, and GSCNP compared with C. The B\textsubscript{max} of GABA\textsubscript{B} receptor assay of GCNP showed a significant decrease (P<0.01) compared with PHNT. SCNP and GSCNP also showed a significant decrease (P<0.001) compared with PHNT. The B\textsubscript{max} of 5-HT\textsubscript{2A} receptor binding study showed a significant decrease (P<0.001) in GCNP, SCNP, and GSCNP compared with PHNT. There was no significant change in the K\textsubscript{d} values of any groups in either receptor study (Table I).

Real-Time PCR Analysis of GABA\textsubscript{B}, 5-HT\textsubscript{2A}, NF-\kappa B, TNF-\alpha, Akt-1, Bax, Caspase-8, and Phospholipase C mRNA in the Brainstem of Experimental Rats

Gene expression studies of GABA\textsubscript{B} and 5-HT\textsubscript{2A} receptors mRNA in the brainstem of PHNT, GCNP, SCNP, and GSCNP showed a significant decrease (P<0.001) compared with C. There was also a significant decrease (P<0.001) in both receptors’ gene expression of GCNP, SCNP, and GSCNP compared with PHNT (Figs. 1, 2). The gene expressions of NF-\kappa B, TNF-\alpha, and Akt-1 mRNA showed a significant increase (P<0.001) in PHNT, GCNP, SCNP, and GSCNP compared with C. The expression of these neuronal survival factors in all treatment groups showed a significant increase (P<0.001) compared with PHNT (Figs. 3–5).

Neuronal apoptosis was studied by observing the gene expression patterns of Bax, caspase-8, and phospholipase C. The expressions of these three genes were significantly downregulated (P<0.001) in all partially hepatectomized rats with and without nanoparticle treatment, compared with PHNT, GCNP, SCNP, and GSCNP showed a significant decrease (P<0.001) compared with C. There was also a significant decrease (P<0.001) in both receptors’ gene expression of GCNP, SCNP, and GSCNP compared with PHNT (Figs. 1, 2).

Statistical Analysis

Statistical evaluations used analysis of variance (ANOVA) in GraphPad Instat (version 2.04a; GraphPad, San Diego, CA). Student Newman-Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made in Sigma Plot (version 2.03). Relative Quantification software was used for analyzing real-time PCR results.

<table>
<thead>
<tr>
<th>Group</th>
<th>GABA\textsubscript{B} B\textsubscript{max} (fmoles/mg protein)</th>
<th>GABA\textsubscript{B} K\textsubscript{d} (nM)</th>
<th>5-HT\textsubscript{2A} B\textsubscript{max} (fmoles/mg protein)</th>
<th>5-HT\textsubscript{2A} K\textsubscript{d} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>82.5 ± 1.4</td>
<td>1.3 ± 0.11</td>
<td>125.3 ± 1.4</td>
<td>1.3 ± 0.10</td>
</tr>
<tr>
<td>PHNT</td>
<td>65.0 ± 2.8\textsuperscript{b}</td>
<td>1.4 ± 0.05</td>
<td>103.0 ± 1.1\textsuperscript{b}</td>
<td>1.2 ± 0.13</td>
</tr>
<tr>
<td>GCNP</td>
<td>52.3 ± 2.3\textsuperscript{b,d}</td>
<td>1.6 ± 0.11</td>
<td>79.0 ± 0.9\textsuperscript{b,e}</td>
<td>1.1 ± 0.11</td>
</tr>
<tr>
<td>SCNP</td>
<td>46.1 ± 2.3\textsuperscript{b,e}</td>
<td>1.5 ± 0.08</td>
<td>70.5 ± 1.0\textsuperscript{b,e}</td>
<td>1.2 ± 0.08</td>
</tr>
<tr>
<td>GSCNP</td>
<td>30.3 ± 1.4\textsuperscript{b,e}</td>
<td>1.8 ± 0.29</td>
<td>41.1 ± 1.1\textsuperscript{b,e}</td>
<td>1.0 ± 0.06</td>
</tr>
</tbody>
</table>

\textsuperscript{a}C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. B\textsubscript{max} represents the number of receptors and K\textsubscript{d} represents the affinity of receptors toward the ligand.

Values are mean ± SEM of five separate experiments.

\textsuperscript{b}P < 0.001 compared with C.

\textsuperscript{c}P < 0.001 compared with PHNT.

\textsuperscript{d}P < 0.01 compared with PHNT.

Fig. 1. Real-time PCR amplification of GABA\textsubscript{B} mRNA in the brainstem of experimental rats. Values are mean ± SEM of five separate experiments. Each group consists of five rats. \textsuperscript{a}P < 0.001 compared with C. \textsuperscript{b}P < 0.001 compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.
with Caspase-8 gene expressions in all the treatment groups were significantly decreased ($P < 0.001$) compared with PHNT. Phospholipase C gene expression was significantly down regulated in GCNP and SCNP ($P < 0.01$) and also in GSCNP ($P < 0.001$) compared with PHNT.

**GABA$_B$ and 5-HT$_{2A}$ Receptor Antibody Staining in the Brainstem of Experimental Rats Using Confocal Microscopy**

GABA$_B$ and 5-HT$_{2A}$ receptor staining using receptor-specific primary antibody and fluorescence-labeled secondary...
antibody showed a significant change in all the groups. There was a significant decrease \((P < 0.001)\) in the pixel intensity of brainstem sections with GABA\(_\text{B}\)- and 5-HT\(_2\text{A}\)-stained receptors in GCNP, SCNP, and GSCNP compared with C. There was a significant decrease in GABA\(_\text{B}\) \((P < 0.05)\) and 5-HT\(_2\text{A}\) \((P < 0.01)\) receptors in PHNT compared with C. The GABA\(_\text{B}\) receptors viewed were significantly decreased \((P < 0.001)\) in GCNP, SCNP, and GSCNP compared with PHNT (Table II, Fig. 9). The 5-HT\(_2\text{A}\) receptors were decreased in GCNP \((P < 0.05)\), SCNP \((P < 0.01)\), and GSCNP \((P < 0.001)\) compared with PHNT (Table III, Fig. 10).

**Narrow Beam Walk Test**

Liver injury leads to changes in motor activity of the animal. The time taken for the rat to cover the entire beam showed a significant increase \((P < 0.001)\) in PHNT, GCNP, SCNP, and GSCNP compared with C and a significant decrease \((P < 0.001)\) in GCNP and SCNP compared with PHNT. The time taken for the rats in

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>52.0 ± 1.7</td>
</tr>
<tr>
<td>PHNT</td>
<td>45.6 ± 1.4(^c)</td>
</tr>
<tr>
<td>GCNP</td>
<td>32.6 ± 1.4(^{b,d})</td>
</tr>
<tr>
<td>SCNP</td>
<td>33.7 ± 1.4(^{b,d})</td>
</tr>
<tr>
<td>GSCNP</td>
<td>25.3 ± 1.1(^{b,d})</td>
</tr>
</tbody>
</table>

\(^a\)C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. Values are mean ± SEM of five separate experiments.

\(^b\)P < 0.001 compared with C.

\(^c\)P < 0.05 compared with C.

\(^d\)P < 0.001 compared with PHNT.
GSCNP showed a significant decrease ($P < 0.01$) compared with PHNT (Fig. 11).

**DISCUSSION**

The functional relationship between the liver and the brain has been known for centuries. Butterworth (1995) reported that neurotransmission in the brain is altered in liver diseases. A spectrum of neuropsychiatric abnormalities in patients with liver dysfunction were observed and was characterized by intellectual impairments, personality changes, and a depressed level of consciousness associated with multiple neurotransmitter systems, cerebral perfusion, and astrocyte dysfunction (Avraham et al., 2009). Jain et al. (1991) reported an onset of mitochondrial damage in brain resulting from decreased synthesis of glutathione by damaged liver, which is the major glutathione synthesis site.

Jain et al. (1991) reported an onset of mitochondrial damage in brain resulting from decreased synthesis of glutathione by damaged liver, which is the major glutathione synthesis site.

The liver has a remarkable capacity to regenerate after cellular damage or tissue removal. Liver regeneration is mostly the result of increased mitosis of hepatocytes. Agonists of GABA can act at the GABA receptor complex, and increased concentrations of the agonists are found in the brainstem in liver failure (Basile and Jones, 1994). Neurosteroids produced in the brain during acute liver failure lead to increased GABAergic tone (Akhoucha et al., 2012a), and elevated intracerebral concentrations of GABA significantly decrease ornithine decarboxylase activity in the liver (Lapinjoki et al., 1983). This is an index for decreased liver cell proliferation and function. There is also an interesting report suggesting that 5-HT can potentially contribute to liver tissue hypoperfusion following hepatic ischemia and reperfusion (Murata et al., 2003).

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**TABLE III. Confocal Imaging Studies of 5-HT$_{2A}$ Receptors in the Brainstem of Experimental Groups**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>46.3 ± 1.4</td>
</tr>
<tr>
<td>PHNT</td>
<td>38.2 ± 1.3$^c$</td>
</tr>
<tr>
<td>GCNP</td>
<td>31.3 ± 2.0$^{a,e}$</td>
</tr>
<tr>
<td>SCNP</td>
<td>28.1 ± 1.6$^{b,e}$</td>
</tr>
<tr>
<td>GSCNP</td>
<td>24.7 ± 1.4$^{b,d}$</td>
</tr>
</tbody>
</table>

$^a$C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. Values are mean ± SEM of five separate experiments.  

$^b$P < 0.001 compared with C.  

$^c$P < 0.01 compared with C.  

$^d$P < 0.001 compared with PHNT.  

$^e$P < 0.01 compared with PHNT.  

$^f$P < 0.05 compared with PHNT.
Sympathetic innervation is important for liver regeneration (Kiba et al., 1995). Our previous study showed increased DNA and protein syntheses, which are cell division markers, in the regenerating liver of GCNP, SCNP, and GSCNP compared with PHNT after 24 hr posthepatectomy (Shilpa et al., 2012a,b). During liver injury, ammonia metabolism is disturbed, leading to a condition called hyperammonemia. Hyperammonemia has been suggested to induce enhanced brainstem ammonia uptake and subsequent glutamine and GABA synthesis and accumulation. The changes in brain glutamate and GABA could be related to altered ammonia metabolism (Dejong et al., 1992). An increased level of ammonia leads to neuronal damage and alteration in the cardiovascular and respiratory centers in the brainstem (Saul et al., 2010). The autonomic regulation of GABA was mediated through GABAB receptors (Sved and Sved, 1990), and reduction in the GABA neurotransmission in the brain regions enhanced DNA synthesis in liver by facilitating the sympathetic tone (Biju, 2000; Biju et al., 2002). The reestablishment of ammonia metabolism in the body and GABA signaling in the brain as a result of liver cell proliferation was clearly studied by observing the reduced GABA<sub>H</sub> receptor expression in the treatment groups. GABA is an inhibitory neurotransmitter and an increased GABAergic neurotransmission is observed

Fig. 10. Confocal image of 5-HT<sub>2A</sub> receptors in the brainstem of control and experimental rats using immunofluorescent 5-HT<sub>2A</sub> receptor specific primary antibody and Alexa Fluor 594 as secondary antibody (arrows). A: Sham-operated control. B: Partially hepatectomized group with no treatment. C: Partially hepatectomized group treated with GABA chitosan nanoparticle. D: Partially hepatectomized group treated with 5-HT chitosan nanoparticle. E: Partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 11. Time taken by the experimental animals in narrow beam walk test. Values are mean ± SEM of five separate experiments. Each group consists of five rats. *P < 0.001, **P < 0.01 compared with C. *P < 0.001 compared with PHNT. C, sham-operated control; PHNT, partially hepatectomized group with no treatment; GCNP, partially hepatectomized group treated with GABA chitosan nanoparticle; SCNP, partially hepatectomized group treated with 5-HT chitosan nanoparticle; GSCNP, partially hepatectomized group treated with GABA and 5-HT chitosan nanoparticle.
during acute liver injury. Alterations of astrocytic–neuronal cross-talk affect brain function. In acute liver failure, astrocytes undergo swelling, which results in increased intracranial pressure and may lead to brain herniation (Akboucha and Butterworth, 2007). Thus GABA and 5-HT chitosan nanoparticle treatment provided hope in rescuing the liver-injured animal. During active hepatocyte proliferation, the 5-HT receptor number was decreased in the brains of partially hepatectomized rats (Sudha, 1997). During liver injury and hepatic insufficiency, the aromatic amino acid catabolism was altered. Thus the plasma levels of aromatic amino acids increase, and they enter the brain. The aromatic amino acid tryptophan enhanced 5-HT synthesis in the brain, which leads to active 5-HT-mediated neurotransmission. As hepatic cell recovery progresses, the aromatic amino acid metabolism also was reactivated. Thus the 5-HT content is decreased in brain (Dejong et al., 1992). The neurotransmitter 5-HT has a profound effect on the control of sleep and mood fluctuations, so excess 5-HT activity in the brain could be responsible for impaired consciousness during liver failure. Our study reported the efficiency of GABA and 5-HT chitosan nanoparticle treatment in reducing the 5-HT receptor expression and signaling in brainstem, which was a good sign of recovery. In our results, the GABA\(_B\) and 5-HT\(_2\)A receptor expression was downregulated in GCNP and SCNP and further decreased in GSCNP compared with PHNT, which supports the previous reports regarding the regulation of neurotransmission mediated through GABA and 5-HT receptors during liver regeneration. Thus, decrease in GABA and 5-HT receptors numbers was a homeostatic adjustment by the brainstem to activate the sympathetic innervation, thereby elevating DNA synthesis in the liver.

During any injury to the organs, a stress-mediated tissue degenerative signaling is activated in the brain. It was more prominent in the case of liver injury. Thus, mechanisms have been developed by the animal’s own body to alleviate the effect of stress by activating neuronal survival molecules. Active liver regeneration, which is supported by increased neuronal survival helps the partially hepatectomized rats to recover easily, with less brain damage. NF-\(\kappa\)B is expressed in diverse cell types in the nervous systems (Neill and Kaltschmidt, 1997). An involvement of NF-\(\kappa\)B in neuronal development demonstrates its activation in neurons in certain regions of the brain during neurogenesis. Inhibition of NF-\(\kappa\)B by an inhibitor such as diethylthio-carbamate (DDTC) was shown to increase cell death and infarct size following transient ischemic insult in rats, suggesting that NF-\(\kappa\)B induces survival signaling in neuronal cells (Hill et al., 2001). NF-\(\kappa\)B is a heterodimer transcription factor that is sequestered in the cytoplasm by an anchor protein, inhibitor of NF-\(\kappa\)B (I\(\kappa\)B). Phosphorylation of NF-\(\kappa\)B on serines 32 and 36 by I\(\kappa\)B kinase leads to its ubiquitination and degradation by proteosomal enzymes, which allows NF-\(\kappa\)B heterodimer to translocate to the nucleus and regulate gene expression. In GCNP, SCNP, and GSCNP, the NF-\(\kappa\)B level was increased compared with PHNT, which showed an increased neuronal survival and maintenance. One well-studied pathway that leads to NF-\(\kappa\)B activation is the involvement of the cytokine TNF-\(\alpha\) through intracellular signaling molecules, TNF receptor-associated factors (TRAF2 and TRAF6), and activated NF-\(\kappa\)B-inducing kinase (NIK), which phosphorylates the IKKs (Karim and Ben-Neriah, 2000). IKK can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNF-\(\alpha\) and platelet-derived growth factor (PDGF)-induced NF-\(\kappa\)B activation has been reported to require Akt (Burow et al., 2000). Also, studies show that NF-\(\kappa\)B appears to be a target of the antiapoptotic Ras/PI3K/Akt pathway, and the expression and activity of Akt is regulated by NF-\(\kappa\)B (Meng et al., 2002). The interrelated activation of NF-\(\kappa\)B, TNF-\(\alpha\), and Akt-1 was required for the survival of neurons in the brainstem.

The differential patterns of localization of TNF-\(\alpha\) receptors in neuronal and glial cells, their state of activation, and the downstream effectors all are thought to play an important role in determining whether TNF-\(\alpha\) will exert a beneficial or harmful effect on the CNS (Figiel, 2008). Even though the neurodegenerative activity of TNF-\(\alpha\) has been documented in many studies, several reports emphasize the neuroprotective role of the same. With a rat ischemia model, Hurtado and colleagues (2001, 2002) demonstrated that TACE (an enzyme required for the activation of TNF-\(\alpha\)) is upregulated after ischemic brain damage and that the increase in TACE expression contributes to a rise in TNF-\(\alpha\) and a subsequent neuroprotective effect after excitotoxic stimuli. TNF-\(\alpha\) induces neuroprotection against excitotoxic damage in primary cortical neurons via sustained NF-\(\kappa\)B activation (Dolga et al., 2008). The report of Larsen and Wendon (2002) explains clearly that inflammatory mediators such as TNF-\(\alpha\) and neuronal survival factors such as NF-\(\kappa\)B and Akt-1 play an important role in the regulation of brain function by liver. Thus, based on the evidence from these studies, irrespective of the neurodegenerative effect, TNF-\(\alpha\) has a neuron-protective and survival effect along with NF-\(\kappa\)B and Akt-1 during liver cell proliferation. From our study, in group GSCNP, a better neuron-survival signaling mechanism in the brainstem was observed compared with PHNT. Ankar-crona et al. (1995) explained that, during brain injury, glutamate accumulation leads to overstimulation of postsynaptic glutamate receptors with intracellular Ca\(^{2+}\) overload and neuronal cell death. Overstimulation of neurotransmitter glutamate was decreased by treatment with GABA and 5-HT in a Parkinson’s disease rat model (Nandhu et al., 2011). Thus, in our study, the combined effect of GABA and 5-HT treatment in GSCNP showed an increased expression of neuronal survival factors and resulted in reduced cell death.

Apoptosis of cells in the brainstem was clarified by studying the gene expression of caspase-8 and Bax. Neuronal apoptosis is achieved by two major apoptotic pathways (extrinsic and intrinsic). The extrinsic pathway involves the binding of cytokines to death receptors, activation of caspase-8 and cleavage and activation of effector caspase-3 (Ashkenazi and Dixit, 1998). Caspase-8 is a key factor uniquely associated with this pathway. The intrinsic
pathway involves translocation of Bax protein from the cytosol to the outer mitochondrial membrane, where it increases membrane permeability and promotes release of cytochrome c, which binds with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, resulting in its cleavage to form activated caspase-9 (Green and Reed, 1998). The JNK family of mitogen-activated protein kinase (MAPK) pathway has been observed to play a central role in both of these apoptotic pathways (Dhanasekaran and Reddy, 2008). MAPK can be phosphorylated by mitogens binding to G-protein-coupled receptors (Yagle et al., 2001). Because of the disturbed ammonia and aromatic amino acid metabolisms in partially hepatectomized rats, GABA and 5-HT contents in the brain are increased, which in turn elevates their receptor-mediated signaling and MAPK phosphorylation. JNKs activate apoptotic signaling either through the upregulation of proapoptotic genes via the transactivation of specific transcription factors including c-Jun or through directly modulating the activities of mitochondrial pro- and antiapoptotic proteins through phosphorylation events. There is also evidence for crosstalk between the Bax-regulated intrinsic mitochondrial pathway and the extrinsic death receptor pathway, in which activated caspase-8 is a key enzyme (Li et al., 1998).

There is evidence explaining how that increase in GABA and 5-HT contents activates apoptotic signaling. Anju et al. (2011) reported that a trigger in the altered transcription of GABA_{B} receptors can be related to activation of the apoptotic pathways by activating Bax expression. 5-HT also activates the mitogen-activated protein kinase, which can influence cell apoptosis (Watts, 1996). Active liver cell proliferation regains the capacity to metabolize aromatic amino acids and ammonia. Thus, decreased expression of GABA and 5-HT in the brainstem suppresses the MAPK phosphorylation and increases neuronal survival in partially hepatectomized rats. In our study, caspase-8 and Bax expression were decreased in all the treatment groups compared with PHNT. Active caspase-8 further activates other caspases such as caspase-3 and results in DNA fragmentation (Kuwana et al., 1998). Thus, reduction in caspase-8 expression showed decreased cell death in nanoparticle-treated rats. Phospholipase C catalyzes the hydrolysis of phosphatidylinositol (4,5)-bisphosphate, resulting in the production of diacylglycerol (DAG) and IP_{3}, which activate protein kinase C (PKC). Phospholipase C (PLC) is the enzyme involved in the synthesis of IP_{3}, so the increased levels of IP_{3} and PLC result in enhanced apoptosis (Szalai et al., 1999). The activators of inhibitory subunit of G-protein receptors significantly suppressed the accumulation of inositol-1-phosphate and inositol-1,4,5-triphosphate in cells (Ohmori and Kuriyama, 1989). IP_{3} receptor inactivation phenotypically mimics Bax deficiency by attenuating caspase-3 expression and activation (Prasitsilp et al., 2000). From our study it was clear that the gene expression of caspase-8, Bax, and phospholipase C were decreased significantly in GCNP and SCNP and further decreased in GABA and 5-HT chitosan nanoparticle-treated groups compared with PHNT, which showed a reduction in neuronal apoptosis in brainstem. The apoptosis and neuronal functional instability resulting from disturbed metabolism and storage capabilities after liver injury suppress many motor activities of the animal. The narrow beam walk test gave a vivid idea about the time spent to traverse the entire length of the rod by the partially hepatectomized rats with no treatment. Motor activity was regained by the animals treated with GABA and 5-HT chitosan nanoparticles individually and in combination. Thus the time taken to traverse the entire length of the rod is decreased in the treated groups.

Hepatocyte proliferation is promoted at the expense of liver function. This was supported by Tanaka et al. (1999), who found that functional regeneration measured by 99mTc-GSA scintigraphy was impaired, compared with the volumetric regeneration in patients who had undergone extensive liver resection. With the loss of active liver cells, the metabolism and function of all body organs are disturbed. From our previous study, uptake of [3H]thymidine and [3H]leucine, which are the markers for DNA and protein syntheses, was significantly increased in the treatment groups compared with the untreated group. This clearly shows an increase in hepatic mass after partial hepatectomy, which further relates to an increase in liver function (Shilpa et al., 2012a,b). Impaired ammonia and aromatic amino acid metabolisms and the effect of neurosteroids such as allopregnanolone (Ahboucha et al., 2006) and dehydroepiandrosterone sulfate (Ahboucha et al., 2012b) contribute to increased GABAergic tone in hepatic encephalopathy. Abrasive changes in GABA and 5-HT neurotransmission furthermore lead to coma, mood alterations, lack of consciousness, and disturbances in cardiovascular and respiratory centers of brainstem. As liver regeneration progresses, the brain regains normal functional capacity. The motor control deficit observed in the untreated partially hepatectomized rats was regained to a great extend in GCNP, SCNP, and GSCNP. This emphasizes the liver and brain function recovery in the present study.

Brain and liver functions are interrelated. The present work reveals the potential for GABA and 5-HT chitosan nanoparticle treatment in improving neuronal survival and reducing neuronal apoptosis in the brainstem after partial hepatectomy in rats. We propose that this supplementation in combination will have better effects against the neuronal loss in brainstem than individual treatment during liver injury. It was evident that GABA and 5-HT chitosan nanoparticle treatment in partially hepatectomized rats provides neuronal protection that will have therapeutic significance in the management of liver-based diseases.

**REFERENCES**


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Molecular and cellular pharmacology

Gammar aminobutyric acid B and 5-hydroxy tryptamine 2A receptors functional regulation during enhanced liver cell proliferation by GABA and 5-HT chitosan nanoparticles treatment

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ABSTRACT

Liver is one of the major organs in vertebrates and hepatocytes are damaged by many factors. The liver cell maintenance and multiplication after injury and treatment gained immense interest. The present study investigated the role of Gamma aminobutyric acid (GABA) and serotonin or 5-hydroxytryptamine (5-HT) coupled with chitosan nanoparticles in the functional regulation of Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptors mediated cell signaling mechanisms, extend of DNA methylation and superoxide dismutase activity during enhanced liver cell proliferation. Liver injury was achieved by partial hepatectomy of male Wistar rats and the GABA and 5-HT chitosan nanoparticles treatments were given intraperitoneally. The experimental groups were sham operated control (C), partially hepectomised rats with no treatment (PHNT), partially hepatectomised rats with GABA chitosan nanoparticle (GCNP), 5-HT chitosan nanoparticle (SCNP) and a combination of GABA and 5-HT chitosan nanoparticle (GSCNP) treatments. In GABA and 5-HT chitosan nanoparticle treated group there was a significant decrease (P < 0.001) in the receptor expression of Gamma aminobutyric acid B and a significant increase (P < 0.001) in the receptor expression of 5-hydroxy tryptamine 2A when compared to PHNT. The cyclic adenosine monophosphate content and its regulatory protein, presence of methylated DNA and superoxide dismutase activity were decreased in GSNP, SCNP and GSNP when compared to PHNT. The Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptors coupled signaling elements played an important role in GABA and 5-HT chitosan nanoparticles induced liver cell proliferation which has therapeutic significance in liver disease management.

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1. Introduction

Liver is an important organ responsible for storage, metabolism and synthesis of major compounds in the body (Rubin and Farber, 1999). Among all organs, the liver has the ability to repair itself after suffering loss of tissue mass. Therefore faster liver regeneration with healthy hepatocytes helps the animals for effective recovery from the metabolic break (Corbin et al., 2003).

Gamma aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the vertebrate central nervous system. The cell proliferative role of GABA is observed in different regions of the body including the development of outer retina in rabbits (Messersmith and Redburn, 1993), leydig cell multiplication in testis (Geigerseder et al., 2004) and promotes neuronal cell proliferation and migration (Ben-Yaakov and Golan, 2003). Biju et al. (2002) reported that baclofen, a GABA agonist, induced epidermal growth factor mediated DNA synthesis in hepatocytes in vitro. The cell proliferation is initialized by the activation of cyclic adenosine monophosphate (cAMP) regulated transcription factors in the regenerating liver (Diehl and Rai, 1996).

Serotonin or 5-hydroxytryptamine (5-HT) has been shown to be mitogenic in many cells, exerting its effect through receptor mediated second messenger pathways. The serotonin 2 receptor has been shown to have mediated cell growth in fibroblasts (Van Obbergen-Schilling et al., 1991). The serotonin 2 receptors are coupled to phospho-inositide turnover and diacylglycerol formation, which activates protein kinase C, an important second messenger for cell division (DeCourcelles et al., 1985). Balasubramanian and Paulose (1998) reported that serotonin can act as a potent co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes.

The use of nanotechnology in the field of medicine could revolutionize the way we detect and treat the damage to human body and disease in the future. Chitosan, deacetylated chitin, is a copolymer of...
2. Materials and methods

2.1. Chemicals used and their sources

Biochemicals and Tri-reagent kit were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [3H] Baclofen (Sp. Activity 42.9 Ci/mmol) and [3H] ketanserin (Sp. Activity 63.3 Ci/mmol) were purchased from Amersham Life Science, UK. Chitosan (MW–25KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

2.2. Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 h light and 12 h dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimize animal suffering. Each group consisted of 6–8 animals. Sham operated control (C), partially hepatectomised group without any treatment (PHNT), partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP), partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP) and partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP) were the five experimental groups. Chitosan nanoparticles modified with drugs, could be recognized by their respective receptors on cells and were transferred into hepatocytes via receptor mediated endocytosis. This enhanced their ability to target to the liver, in which receptor mediated cell signaling was activated, and enabled the longevity of these nanoparticles in the liver. In contrast, chitosan nanoparticles without modification, targeted sparsely to the liver, and a large part of these nanoparticles were cleared from the body in urine (Tian et al., 2010; Park et al., 2007). So giving importance to the above fact and based on our previous observations, a control group treated with chitosan nanoparticle alone was not included.

2.3. Experimental procedures

2.3.1. Preparation of GABA and 5-HT chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method (Calvo et al., 1997). The incorporation of GABA and 5-HT into chitosan nanoparticles individually and in combination, standardization of encapsulation efficiency and in vitro release profile studies were done according to Shilpa et al. (2012a, 2012b). The nanoparticles were washed thoroughly and were dispersed in saline.

2.3.2. Partial hepatectomy and treatment

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16 h fast (Higgins and Anderson, 1931). Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M. to avoid diurnal variations in responses. After surgery, 1 ml of 30 μg/μl GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and a combination of GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats. The rats were killed by decapitation 24 h post hepatectomy and liver was dissected out quickly and kept over ice. The tissues were stored at −80 °C until assayed.

2.3.3. Quantification of cAMP

The liver from each experimental group was homogenized in a polytron homogenizer in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 g for 15 min and the supernatant was transferred to fresh tubes for cAMP assay using [3H] cAMP Biotrak Assay System kit. The unknown concentrations were determined from the standard curve using appropriate dilutions and calculated for pmoles/mg protein. Cmax was plotted on the y-axis against picomoles of inactive cAMP standards on the x-axis of a linear graph paper, where Cmax is the counts per minute bound in the absence of unlabeled cAMP and C0 is the counts per minute bound in the presence of standard or unknown unlabeled cAMP. From the Cmax/C0 value for the sample, the number of picomoles of unknown cAMP was calculated. Protein was measured according to Lowry et al. (1951) using bovine serum albumin as standard. The intensity of the purple blue color formed was proportional to the amount of protein which was read in a spectrophotometer at 660 nm.

2.3.4. Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors binding studies using [3H] baclofen and [3H] ketanserin

[3H] Baclofen binding to GABA receptor in the membrane preparations were assayed (Hills et al., 1987). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.4 containing 2 mM CaCl2 and 0.3–0.4 mg protein. In saturation binding experiments, 10–100 nM of [3H] baclofen was incubated with and without excess of 100 μM unlabeled baclofen. The incubations were carried out at 20 °C for 20 min. The binding reactions were terminated by centrifugation at 14000 g for 10 min. The dried pellet was resuspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

[3H] Ketanserin binding to 5-hydroxy tryptamine 2A receptor in the crude synaptic membrane preparation was done according to the modified procedure of Leysen et al. (1982). Crude membrane preparation was suspended in 50 mM Tris sulfate buffer, pH 7.6 containing 0.3–0.4 mg protein. In saturation binding experiments, assays were done using different concentrations of 0.5–10 nM of [3H] ketanserin which was incubated with and without

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excess of unlabeled 10 μM ketanserin. Tubes were incubated at 37 °C for 15 min and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 mL of ice cold 50 mM Tris sulfate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The receptor binding parameters were determined using Scatchard analysis (Scatchard, 1949). The specific binding was determined by subtracting non-specific binding from the total binding. The binding parameters, maximal binding (Bmax) and equilibrium dissociation constant (Kd), were derived by linear regression analysis by plotting the specific binding of the radioligand on x-axis and bound/free on y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The Kd is inversely related to receptor affinity.

2.3.5. Superoxide dismutase assay
The liver from each experimental group was homogenized in a buffer containing 10 mM EDTA, 50 mM Tris–HCl, pH 8.2 and centrifuged at 40000g for 15 min. The protein content of whole supernatant of the total homogenate was estimated (Lowry et al., 1951). SOD was analyzed after the inhibition by SOD of the pyrogallol autoxidation (Marklund and Marklund, 1974) at pH 8.2. Briefly, 1 ml reaction mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris–HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 min with or without 250 μg enzyme protein. The inhibition of pyrogallol was linear with the activity of the enzyme present. Fifty percent inhibition/(mg protein min⁻¹) was considered as one unit of enzyme activity.

2.3.6. Analysis of gene expression by real-time polymerase chain reaction
PCR analyses were conducted with gene-specific primers and fluorescently labeled Taqman probe of Gamma aminobutyric acid B, 5-hydroxy tryptamine 2A, CAMP regulatory element binding protein (CREB), MAT2A, SOD and hepatocyte growth factor (HGF) which were designed by Applied Biosystems. Endogenous control, β-actin, was labeled with a report dye, VIC.

RNA was isolated from the liver of experimental rats using the Tri-reagent according to the procedure of Chomczynski and Sacchi (1987). Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 μL contained 0.2 μg total RNA, 10X RT buffer, 25X dNTP mixture, 10 X random primers, MultiScribe RT (50 U/μL) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time-PCR assays were performed in 96-well plates in an ABI 7300 Real-time-PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 μL contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for assay on demand and endogenous control β-actin and 12.5 μL of Taqman 2 × Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNase free water. The following thermal cycling profile was used (40 cycles): 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples (ΔCT = CT Target−CTβ-actin).

It was further normalized with the control (ΔΔCT = ΔCT−CT Control). The fold change in expression was then obtained as (2−ΔΔCT) and the graph was plotted using log 2−ΔΔCT.

2.3.7. DNA methylation study
The DNA was isolated from the liver of experimental rats using TRI reagent according to the procedure of Chomczynski (1993). DNA concentration was determined by ultraviolet spectrophotometry (UV–1700 Pharma Spec, Shimadzu) with absorbance at 260 and 280 nm. All DNA samples had 260 to 280 absorbance ratios ≈1.7. DNA methylation was determined by using the modified method of Balaghi and Wagner (1993), in which DNA is incubated with [3H] methyl S-adenosylmethionine in the presence of the Cpg Methyl transferase. The reaction mixture contained 0.25 μg DNA, 0.015 U CpG Methyl transferase enzyme (product no. M0226S; New England Biolabs, Beverly, MA), [3H] methyl S-adenosylmethionine (80 Ci/mmol, American radiolabeled chemicals, Inc., Saint Louis, USA), 1.5 μl NEB buffer (New England Biolabs, Beverly, MA), and sterile-filtered water to a total reaction volume of 15 μL. The mixture was incubated at 30 °C for 1 h and placed on ice for 5 min. The reaction mixture were loaded onto a 2.5 cm, round, Whatman DE81 ion-exchange paper filter. The filter was washed successively three times with 7.5 ml of 0.5 M sodium phosphate buffer (pH 8.0), then with 1 ml 70% ethanol, and finally with 1 ml 100% ethanol. The filter was dried at room temperature and the radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The ability of DNA to incorporate [3H] methyl groups in vitro is inversely related to endogenous DNA methylation.

2.3.8. Immunohistochemical analysis by confocal microscope
The experimental rats were deeply anesthetized and was transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the liver from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 h and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 μm liver sections were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked for 1 h with PBS containing 5% normal goat serum and 0.1% triton X-100. The primary antibodies of Gamma aminobutyric acid B (1500 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) and 5-hydroxy tryptamine 2A (1:1000 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the liver slices were rinsed with PBS and then incubated with fluorescent labeled secondary antibody (Alexa Fluor 594, code-A10102) prepared in PBS with 5% normal goat serum and 0.1% triton X-100 at 1:1000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using confocal imaging system (Leica SP 5). Quantification was done using Leica application suit advanced fluorescence (LASAF) software by considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

2.3.9. Statistical analysis
Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a, San Diego, USA). Student Newman–Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made using...
SIGMA PLOT (Ver 2.03). Relative Quantification Software was used for analyzing Real-Time PCR results.

3. Results

3.1. Analysis of Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptor studies

Gamma aminobutyric acid B receptor and 5-hydroxytryptamine 2A receptor subtypes mediated liver cell multiplication in partially hepatectomised rats were studied. $B_{\text{max}}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand. $B_{\text{max}}$ of Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptor study showed a significant decrease ($P < 0.001$) for groups PHNT, GCNP, SCNP and GSCNP when compared to C. The $B_{\text{max}}$ of Gamma aminobutyric acid B receptor was significantly decreased in GCNP ($P < 0.001$) and SCNP ($P < 0.01$) when compared to PHNT. The 5-hydroxytryptamine 2A receptor $B_{\text{max}}$ was significantly increased in GCNP ($P < 0.05$) and SCNP ($P < 0.001$) when compared to PHNT. In GCNP group it was a significant decrease ($P < 0.001$) in the receptor $B_{\text{max}}$ of Gamma aminobutyric acid B receptor and a significant increase ($P < 0.001$) in the receptor $B_{\text{max}}$ of 5-hydroxytryptamine 2A when compared to PHNT. The $K_d$ value of Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptors for PHNT, GCNP and GSCNP showed a significant increase ($P < 0.001$) with respect to C. In SCNP group, $K_d$ value of Gamma aminobutyric acid B receptor showed a significant increase and 5-hydroxytryptamine 2A receptor showed a significant decrease ($P < 0.001$) with that of PHNT. While comparing with PHNT, $K_d$ value of Gamma aminobutyric acid B receptor binding in GCNP, SCNP and GSCNP and 5-hydroxytryptamine 2A receptor binding in SCNP showed a significant decrease ($P < 0.001$) (Table 1).

3.2. Quantification of cAMP

cAMP is involved in G protein coupled receptor mediated cell signaling during liver regeneration. cAMP content of the regenerating liver of PHNT was significantly decreased ($P < 0.001$) compared to C. For groups GCNP, SCNP and GSCNP also the cAMP content was significantly reduced ($P < 0.001$) when compared to C. The cAMP content of groups GCNP and SCNP showed a significant decrease ($P < 0.05$) and group GSCNP also showed a significant decrease ($P < 0.01$) when compared to PHNT (Fig. 1).

3.3. SOD assay

The concentration of antioxidant enzyme SOD depends on the formation of reactive oxygen species in the body. The reactive oxygen species levels were increased due to any kind of damage to the cells, which further led to an increase in the SOD activity also. The SOD activity was significantly decreased ($P < 0.001$) for groups PHNT, G and GCNP when compared to C. There was a significant decrease ($P < 0.001$) in SOD concentration in GCNP, SCNP and GSCNP when compared to PHNT (Table 2).

3.4. Real-time PCR analysis of Gamma aminobutyric acid B, 5-hydroxytryptamine 2A, CREB, MAT2A, SOD and HGF mRNA

Gene expression study of Gamma aminobutyric acid B receptor mRNA in the regenerating liver of PHNT, GCNP, SCNP and GSCNP showed a significant decrease ($P < 0.001$) when compared to C and also a significant decrease ($P < 0.001$) in each of the nanoparticle treated groups when compared to PHNT (Fig. 2).

For 5-hydroxytryptamine 2A receptor, when compared to C the gene expression was observed to be decreased significantly ($P < 0.001$) in all the partially hepatectomised groups with or without nanoparticle treatment. There was no significant change in the expression of the receptor for GCNP when compared to PHNT. Considering the other groups, there was a significant increase in 5-hydroxytryptamine 2A receptor gene expression in SCNP ($P < 0.01$) and GSCNP ($P < 0.001$) when compared to PHNT (Fig. 3).

The gene expression of transcription factor CREB, which was activated by cAMP, involved in the signaling cascade of G protein coupled receptors showed a significant decrease ($P < 0.001$) in PHNT, GCNP, SCNP and GSCNP when compared to C. The gene expression of CREB was decreased significantly ($P < 0.001$) in GCNP, SCNP and GSCNP when compared to PHNT (Fig. 4).

The methionine adenosyltransferase (MAT), which catalyzes the formation of the methyl donor S-adenosylmethionine, helps in DNA methylation. Its gene expression was significantly downregulated.

![Fig. 1. cAMP content in the liver of experimental rats. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. *$P < 0.001$, †$P < 0.01$ with respect to C. ‡$P < 0.001$ and §$P < 0.05$ with respect to PHNT. C-Sham operated control. PHNT–partially hepatectomised group with no treatment. GCNP–partially hepatectomised group with GABA chitosan nanoparticle treatment. SCNP–partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP–partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. $B_{\text{max}}$ represents the number of receptors and $K_d$ represents the affinity of receptors towards the ligand.](http://dx.doi.org/10.1016/j.ejphar.2013.05.028)
Table 2
Superoxide dismutase assay in the liver of experimental rats.

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<td>C</td>
<td>90.7 ± 3.20</td>
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<tr>
<td>PHNT</td>
<td>58.3 ± 1.59</td>
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<tr>
<td>GCNP</td>
<td>29.5 ± 0.89&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>SCNP</td>
<td>27.0 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>GSCNP</td>
<td>19.0 ± 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
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C- Sham operated control, PHNT-partially hepatectomised group with no treatment, GCNP-partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP-partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP-partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

Values are mean ± S.E.M. of 4–6 separate experiments.

<sup>a</sup> P < 0.001 with respect to Sham operated control.

<sup>d</sup> P < 0.001 with respect to PHNT.

Fig. 2. Real-time PCR amplification of GABA<sub>B</sub> mRNA in the liver of experimental rats. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 6–8 rats. *P < 0.001 with respect to C. <sup>★</sup><sup>a</sup><sup>p</sup><sup>o</sup><sup>★</sup><sup>★</sup> when compared to PHNT. C- Sham operated control, PHNT-partially hepatectomised group with no treatment, GCNP-partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP-partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP-partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

regulated (P < 0.001) in GCNP, SCNP and GSCNP when compared to C and PHNT. While considering PHNT, the gene expression was significantly reduced (P < 0.001) when compared to C (Fig. 5).

Superoxide dismutase is an antioxidant enzyme and its expression was directly dependant on the level of reactive oxygen species, which activates the apoptotic signaling. The SOD gene expression was significantly down regulated (P < 0.001) in GCNP, SCNP and GSCNP when compared to C and PHNT. The gene expression of SOD in PHNT was significantly reduced (P < 0.001) when compared to C (Fig. 6).

Hepatocyte growth factor (HGF) is the most potent stimulator of hepatocyte growth and DNA synthesis identified. Thus increase in HGF gene expression was considered as a marker for liver regeneration. HGF gene expression was significantly up regulated (P < 0.001) in GCNP, SCNP and GSCNP when compared to C and PHNT (Fig. 7).

3.5. DNA methylation study

Rapid DNA synthesis prior to mitosis resulted in decreased DNA methylation. Thus in vitro methyl group incorporation to the DNA, which were isolated from rapidly dividing cells, was observed to be high when compared to normal cells. The [3H] methyl group incorporation in the DNA was significantly up regulated (P < 0.001) in GCNP, SCNP and GSCNP when compared to C and PHNT. For PHNT, the incorporation in PHNT was significantly increased (P < 0.001) when compared to C (Fig. 8). To support the experimental outcomes of methylation study, the DNA content was also increased significantly (P < 0.001) in nanoparticle treated group when compared to partially hepatectomised groups with no treatment (Table 3).

3.6. Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors antibody staining in the liver of control and experimental rats using confocal microscope

Gamma aminobutyric acid B and 5-hydroxy tryptamine 2A receptors staining using receptor specific primary antibody and...
fluorescent labeled secondary antibody showed a significant change in receptor expression. There was a significant decrease ($P < 0.001$) in Gamma aminobutyric acid B expression in GCNP, SCNP and GSCNP when compared to C. For PHNT the receptor expression was down regulated significantly ($P < 0.01$) with respect to C. The receptor expression was down regulated significantly in GCNP ($P < 0.05$), SCNP ($P < 0.01$) and GSCNP ($P < 0.01$) when compared to PHNT (Fig. 9; Table 4).

Confocal imaging of 5-hydroxytryptamine 2A receptor expression in the liver of experimental groups showed a significant decrease in PHNT and GCNP ($P < 0.001$) and also a decrease in SCNP and GSCNP ($P < 0.05$) when compared to C. The receptor expression was up regulated significantly in GCNP ($P < 0.01$) and also in SCNP and GSCNP ($P < 0.001$) when compared to PHNT (Fig. 10; Table 5).

4. Discussion

Drug delivery is likely to benefit from the development of nanotechnology. Nanomedicine minimizes the damage to healthy cells in the body (Zajtchuk, 1999). In the last two decades, Chitosan nanoparticles have been extensively explored for pharmaceutical application (Sailaja et al., 2010). Chitosan is a natural and second attractive material for drug delivery. As previously reported, several factors, such as particle size, polymer composition, molecular weight and surface characteristic of nanoparticles determine the particle distribution in the various organs of the body (Van Oss, 1978; Tabata and Ikada, 1989; Gref et al., 1994; Storm et al., 1995; Gao et al., 2004; Cho et al., 2007). The GABA and 5-HT chitosan nanoparticles composition was the main reason for their distinct body distribution over unmodified chitosan nanoparticles.
Liver is a body organ responsible for numerous functions such as storage and metabolism. Liver cell damage occurs in many ways. Alcohol-induced cell death and inflammation result in scarring on liver (Lands, 1995; Maher and Friedman, 1995). An over dose of many drugs, parasite attack (Seydel and Stanley, 1998) and hepatotoxic chemicals (Boobis et al., 1992; Fawthrop et al., 1991) can cause severe liver injury with the potential to progress to liver failure. Due to all these reasons the metabolic functions of liver gets disturbed. So the regeneration of damaged hepatocytes is essential for the balanced routine body functions.

After two-third hepatectomy of liver, the major metabolic pathways were disrupted. Liver starts to regenerate and the maximum DNA synthesis occurs by 24 h post hepatectomy (Kountouras et al., 2001). Our earlier studies explained an enhancement in DNA and protein synthases in hepatocytes, markers of active cell division, in GABA and 5-HT chitosan nanoparticle treated partially hepatectomised rats (Shilpa et al., 2012a, 2012b). For the activation of cell division, GABA and 5-HT receptors functional regulation in various signaling pathways were controlled during liver regeneration. Usually in non replicating tissues (group C), GABA and 5-HT receptors activation and deactivation occurs by endocytosis and exocytosis of receptor on the membrane. Previous report showed that the exposure of Gamma aminobutyric acid B agonist, baclofen, inhibited Schwann cells proliferation due to increased expression of Gamma aminobutyric acid B receptor (Magnaghi et al., 2004). In our results Gamma aminobutyric acid B receptor expression was reduced in groups with GABA and 5-HT chitosan nanoparticle treatment individually and better in combination that supported active hepatocyte proliferation. Activation of 5-hydroxytryptamine 2 receptors in cultured cortical neurons has been shown to inhibit GABA receptor currents via a protein kinase C mediated pathway (Feng et al., 2001). The 5-HT of combination treatment in GSCNP group down regulated Gamma aminobutyric acid B receptor which was an indication of improved hepatocyte proliferation.

During enhanced hepatocyte proliferation, the expression of 5-hydroxytryptamine 2A subtype serotonin receptors in the liver was increased (James and Perkins, 2006). Our study also supported this and observed an up regulation in 5-hydroxytryptamine 2A receptor expression in the treatment groups exposed to 5-HT. Some in vivo and in vitro studies have demonstrated that serotonin exposure resulted in the desensitization and down-regulation of 5-hydroxytryptamine 2A receptors (Shi et al., 2007). The serotonin exposure to the their receptors in SCNP groups is more compared to GSCNP group. Thus 5-hydroxytryptamine 2A receptor expression and activity in GSCNP group was observed to be increased due to reduced receptor desensitization, again pointed the enhancement in liver cell multiplication. The Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptor expression patterns in the experimental groups were further confirmed by confocal microscopy images. Thus treatment with GABA and 5-HT chitosan nanoparticles increased liver cell proliferation by regulating both Gamma aminobutyric acid B and 5-hydroxytryptamine 2A receptors functions.

**Table 3**

DNA content in the liver of experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>DNA content (ng/mg liver weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.64 ± 0.020</td>
</tr>
<tr>
<td>PHNT</td>
<td>0.92 ± 0.010*a</td>
</tr>
<tr>
<td>GCNP</td>
<td>1.06 ± 0.005*a,d</td>
</tr>
<tr>
<td>SCNP</td>
<td>1.08 ± 0.005*a,d</td>
</tr>
<tr>
<td>GSCNP</td>
<td>1.15 ± 0.008*a,d</td>
</tr>
</tbody>
</table>

C-Sham operated control, PHNT—partially hepatectomised group with no treatment, GCNP—partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP—partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP—partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

Values are mean ± S.E.M of 4–6 separate experiments.

*a P < 0.001 with respect to Sham operated control.

*d P < 0.001 respect to PHNT.

**Fig. 9.** Confocal image of Gamma aminobutyric acid B receptors in the liver of control and experimental rats using immunofluorescent Gamma aminobutyric acid B receptor specific primary antibody and Alexa Fluor 594 as secondary antibody. A—negative control, B—Sham operated control (C), C—partially hepatectomised group with no treatment (PHNT), D—partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP), E—partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP) and F—partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP).

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The expression of Gi2 alpha subunit of G protein is increased by 24 h of hepatectomy and thus the activity of adenylyl cyclase is decreased in the S phase of cell cycle (Anna et al., 1992). In the pre-replicative phase of liver regeneration, there is a burst in cAMP-dependent protein kinase A (PKA) activation that is partly down regulated during the phase of active regeneration (S phase). In normal regeneration of hepatocytes the G protein receptor activation occurs in the initial phase of cell division which further leads to a rise in cAMP and PKA levels. But later, during the shift from G1 to S phase, the PKA mediated cell signaling gets suppressed (Ekanger et al., 1989) and promote protein kinase C mediated cell signaling (Arturo, 2003). So cAMP content and CREB expression were reduced in the regenerating livers of PHNT and further reduced significantly in GCNP, SCNP and GSCNP, which promoted enhanced liver cell proliferation in the active phase of cell cycle.

Table 4

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>30.4 ± 1.0</td>
</tr>
<tr>
<td>PHNT</td>
<td>26.8 ± 1.0</td>
</tr>
<tr>
<td>GCNP</td>
<td>22.3 ± 0.8</td>
</tr>
<tr>
<td>SCNP</td>
<td>19.4 ± 0.6</td>
</tr>
<tr>
<td>GSCNP</td>
<td>16.4 ± 0.4</td>
</tr>
</tbody>
</table>

C-Sham operated control, PHNT—partially hepatectomised group with no treatment, GCNP—partially hepatectomised group with GABA chitosan nanoparticle treatment and SCNP—partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP—partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment. Values are mean ± S.E.M of 4-6 separate experiments.

P < 0.01. 

Growth factor stimulation by platelet-derived, epidermal and insulin-like growth factors results in an increase in intracellular reactive oxygen species (Sauer et al., 2001). This reactive oxygen species production can inactivate phosphatases at the cell membrane (Meng et al., 2002), activate kinases and transcription factors (Sauer et al., 2001) leading to cell cycle progression. An increase in reactive oxygen species concentration leads to a decrease in SOD activity (Gajewska and Sklodowska, 2007). Our results also explained a decrease in SOD gene expression and activity in the treatment groups than PHNT, which emphasized an increase in cell proliferation. DNA methylation is a modification of DNA established immediately after DNA synthesis in the ‘S’ phase. In our study, the in vitro methyl group incorporation was significantly high in the treatment groups when compared to C and PHNT. This suggested the occurrence of reduced methylated DNA in the liver of each experimental group with active hepatocyte proliferation. There are studies reporting that in mammalian cells the synthesis and methylation of DNA do not occur simultaneously (Gruenbaum et al., 1983) and when the cells enter the S phase it was found that the apparent decrease in the methylated DNA was resulted due to increased DNA content of the cells resulting from DNA replication (Smith et al., 1980). Decreased methylation in DNA obtained from the regenerating liver that was treated with GABA and 5-HT chitosan nanoparticles clearly explained the increase in DNA content by DNA replication followed by cell mitosis. The MAT2A gene expression, the enzyme synthesis in the GSCNP group was significantly reduced. Thus the extend of DNA methylation in the regenerating liver of GSCNP were less compared to other experimental groups, again projected the significance of GABA and 5-HT chitosan nanoparticles treatment for active hepatocyte DNA synthesis and division. Hepatocyte growth factor (HGF) is an important growth factor which is expressed and elevated during liver regeneration in partially hepatectomised rats and is a potent stimulator of hepatocyte growth and DNA replication. The expression of MAT2A gene leads to elevated DNA content of the cells resulting from DNA replication followed by cell mitosis. MAT2A gene expression, the enzyme synthesis in the GSCNP group was significantly reduced. Thus the extend of DNA methylation in the regenerating liver of GSCNP were less compared to other experimental groups, again projected the significance of GABA and 5-HT chitosan nanoparticles treatment for active hepatocyte DNA synthesis and division. Hepatocyte growth factor (HGF) is an important growth factor which is expressed and elevated during liver regeneration in partially hepatectomised rats and is a potent stimulator of hepatocyte growth and DNA replication.
synthesis identified (Su et al., 2002). HGF is recognized as one of the most important factors in the regulation of liver regeneration after surgical resection or chemical damage (Jiang et al., 1993). The increased expression of HGF gene in the present study supported the concept of therapeutic significance of GABA and 5-HT chitosan nanoparticle induced liver regeneration.

5. Conclusion

Liver is associated with many metabolic functions in the body. Damaged hepatocytes impair the major liver functions and lead to death. So the active proliferation of hepatocytes from damaged liver for the balanced functioning of body is important. Treatment with GABA and serotonin chitosan nanoparticles to partially hepatectomised rat liver improved the cell signaling mechanisms during hepatic regeneration when compared to the regeneration without any treatment. An active antioxidant enzyme system was developed during the treatment that facilitated protection from reactive oxygen species induced hepatocyte damage. It was evident that GABA and 5-HT chitosan nanoparticles treatment in partially hepatectomised rats renders fast hepatocyte proliferation with reduced damage which will have therapeutic significance in the management of liver based diseases.

Acknowledgments

This work was supported by research Grants from DBT, DST, ICMR, Government of India and KSCSTE, Govt. of Kerala to Dr. C.S. Paulose. Shilpa Joy thanks UGC, Government of India for the Maulana Azad Fellowship.

References

Table 5

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>46.7 ± 0.29</td>
</tr>
<tr>
<td>PHNT</td>
<td>30.0 ± 0.20</td>
</tr>
<tr>
<td>GCNP</td>
<td>35.7 ± 0.22</td>
</tr>
<tr>
<td>SCNP</td>
<td>39.8 ± 0.24</td>
</tr>
<tr>
<td>GSCNP</td>
<td>41.9 ± 0.27</td>
</tr>
</tbody>
</table>

C-Sham operated control, PHNT=partially hepatectomised group with no treatment, GCNP=partially hepatectomised group with GABA chitosan nanoparticle treatment, SCNP=partially hepatectomised group with 5-HT chitosan nanoparticle treatment and GSCNP=partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment.

Values are mean ± S.E.M of 4–6 separate experiments.

* P < 0.001.
** P < 0.05 with respect to C.
** P < 0.001.
* P < 0.01 with respect to PHNT.


Tabata, Y., Ikada, Y., 1989. Protein precocating of polylactide microspheres containing a lipophilic immunopotentiator for enhancement of macrophage phagocytosis and activation. Pharm. Res. 6, 296–301.


Increased Cortical Neuronal Survival During Liver Injury: Effect of Gamma Aminobutyric Acid and 5-HT Chitosan Nanoparticles

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Use of nanoparticulate drug delivery system for an effective therapeutic outcome against diseases gains immense hope in the study of drug delivery to partially hepatectomised rats. In the present study, partially hepatectomised rats treated with Gamma aminobutyric acid (GABA) and serotonin (5-HT) chitosan nanoparticles, individually and in combination, were evaluated to analyse their role in GABA B and 5-HT2A receptors functional regulation, interrelated neuronal survival mechanisms by nuclear factor kappa B (NF-κB), tumour necrosis factor-α (TNF-α), Akt-1 and the antioxidant enzyme superoxide dismutase (SOD) in the cerebral cortex. A significant decrease in GABA B and 5-HT2A receptor numbers and gene expressions denoted a homeostatic adjustment by the cerebral cortex to trigger the sympathetic innervations during elevated DNA synthesis in the liver. GABA B and 5-HT2A signalling directly influenced the cyclic AMP response element binding protein (CREB) expression, neuronal survival and ROS mediated cell damage which was confirmed from the gene expression of NF-κB, TNF-α, Akt-1 and SOD. In addition to enhanced hepatocyte proliferation, GABA and 5-HT chitosan nanoparticle treatment protected the neurons from ROS mediated cell damage and promoted their survival in the cerebral cortex. This has application in liver based diseases and treatments with nanosized active compounds.

KEYWORDS: Chitosan Nanoparticles, GABA, Serotonin, Neuronal Survival, Liver Regeneration, Antioxidant System.

INTRODUCTION

Nanoparticulate drug delivery systems provide new hopes in solving problems in the area of drug delivery.1 Nanotechnology is the technology that deals with one billionth of a meter scale.2 To a wide extend, biopolymer conjugated drugs coupled with nanotechnology enhances the stability and pharmacological efficiency of the active compound. Chitosan, deacetylated chitin, is a copolymer of β-(1, 4) linked glucosamine (deacetylated unit) and N-acetyl glucosamine (acyetylated unit).3 Chitosan is non-toxic, biodegradable and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections. Polymeric drug delivery systems can be used to deliver drugs directly to the intended site of action which results in slow release and minimized side effects elsewhere in the body and decrease the long-term use of many drugs.

The liver is a vital organ and has a wide range of functions, which include synthesis, storage, detoxification, metabolism and redistribution of amino acids, carbohydrates, fats, vitamins and proteins. Damage to liver occurs by over consumption of alcohol death, drugs with analgesic and antipyretic action, attack of parasites like Entamoeba histolytica and hepatotoxic chemicals. Patients often develop signs of liver dysfunction in the immediate postoperative period as a result of reduced liver mass, but normal liver function resumes once the removed liver mass is restored4 but not completely in advanced liver disease. The presence of various ligands in the initiation, propagation and termination of the mitotic stimulus, such as priming factors, co-mitogens, growth factors and their suppressors, is necessary for the successful and complete restoration of hepatic mass.5 Prolonged liver dysfunction, such as liver cirrhosis leading to hepatocyte damage, can harm the brain, leading to a serious and potentially fatal brain disorders.
Brain plays an important regulatory role in hepatic functions. The cerebral cortex plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness. In acute liver failure, high ammonia levels raise cerebral blood flow and increased inflammatory response have been identified as major contributors to the development of hepatic encephalopathy and the related brain herniation, coma and brain swelling. This affects neurotransmitter level and its receptor activation in brain. Auto regulation of blood flow in different brain regions is impaired and is associated with anaerobic glycolysis and oxidative stress. Inflammatory mediators like tumour necrosis factor (TNF-α) also play important role in regulation of cortical function by liver. So the regeneration of damaged liver restores the functions of cerebral cortex. The liver is richly innervated and the central thyrotropin-releasing hormone, one of the important peptide transmitter substances, regulates hepatic proliferation through autonomic nervous system. Extirpation of the brain cortex was shown to increase the rate of liver cell proliferation describing that the cortex exerts an inhibitory function on liver cell division, growth and also transection of the spinal cord above the area innervating the liver resulted in decreased DNA synthesis. There are several reports highlighting the regulation of hepatic proliferation by the cerebral cortex, but the role of neurotransmitters and their receptors in mediating neuronal survival during neurotransmitter conjugated chitosan nanoparticle induced liver regeneration are not well studied.

Gamma aminobutyric acid (GABA), a neurotransmitter, is reported to have an inhibitory effect on sympathetic outflow. Brain GABAergic changes are reported to regulate autonomic nerve function in rats. GABA and GABA receptors were widely distributed in mammalian brain and are in high concentration in cortical, hippocampal, thalamic, basal ganglia, and cerebellar structures. In animal models of liver injury, an increase in GABAergic tone has been demonstrated due to both an increase in GABA release and enhanced activation of the GABA receptor complex. Serotonin (5-hydroxytryptamine [5-HT]) is also another neurotransmitter in the central nervous system and peripheral nervous systems and a hormone produced by the gut and transported in platelets. The central serotonergic neurons participate in the regulation of sympathetic nerve discharge and have an inhibitory influence on central sympathetic pathways. 5-HT regulates cell proliferation, migration and maturation in a variety of cell types and alters the cytoskeleton of cells and thus influences the formation of cell contacts. 5-HT has been implicated more in behaviour, physiological mechanisms, and disease processes than any other brain neurotransmitter.

The present study explained the functional regulation of GABA and 5-HT receptors mediated signalling and their role in the activation of neuronal survival factors and antioxidant enzyme in the cerebral cortex during active liver regeneration triggered by GABA and 5-HT chitosan nanoparticles. In this work we tried to elucidate the therapeutic application of GABA and 5-HT chitosan nanoparticles in balancing the non obstructive cortical functions and enhanced liver cell proliferation after partial hepatectomy.

MATERIALS

Chemicals Used and Their Sources

Biochemicals and Tri-reagent kit were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. [3H] baclofen (Sp. Activity 42.9 Ci/mmol) and [3H] ketanserin (Sp. Activity 63.3 Ci/mmol) was purchased from Amersham Life Science, UK. Chitosan (MW-25 KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

Animals

Experiments were carried out on adult male Wistar rats of 250–300 g body weight purchased from Kerala Agricultural University, Mannuthy, India. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water ad libitum. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimize animal suffering. Each group consisted of 6 animals. Sham operated control treated with saline (C), partially hepatectomised rats with no treatment (PHNT), GABA chitosan nanoparticles treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle treatment (GSCNP) were the five experimental groups.

EXPERIMENTAL PROCEDURES

Preparation of GABA and 5-HT Chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method. The incorporation of GABA and 5-HT into chitosan nanoparticles individually and in combination, standardization of encapsulation efficiency and in vitro release profile studies were done according to Shilpa et al. The nanoparticles were washed thoroughly and were dispersed in saline.

Partial Hepatectomy and Sacrifice

Two–thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anesthesia, following a 16 hour fast. Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M to avoid diurnal variations in responses. After surgery, 1 ml of
30 µg/µl GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and a combination of GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats. The rats were sacrificed by decapitation 24 hours post heptectomy and brain cerebral cortex was dissected out quickly and kept over ice according to the procedure of Glowinski and Iversen. The tissues were stored at −80 °C until assayed.

**GABA_β_ and 5-HT_2A_ Receptor Binding Studies in the Cerebral Cortex Using [3H] Baclofen and [3H] Ketanserin**

[3H] Baclofen binding to GABA_β_ receptor in the membrane preparations were assayed. Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.4 containing 2 mM CaCl_2_ and 0.3–0.4 mg protein. In saturation binding experiments, 10–100 nM of [3H] baclofen was incubated with and without excess of 100 µM unlabelled baclofen. The incubations were carried out at 20 °C for 20 minutes. The binding reactions were terminated by centrifugation at 14000 × g for 10 minutes. The dried pellet was re-suspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

[3H] Ketanserin binding to 5-HT_2A_ receptor in the crude synaptic membrane preparation was done according to the modified procedure of Leysen et al. Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.6 containing 0.3–0.4 mg protein. In saturation binding experiments, assays were done using different concentrations of 0.5–10 nM of [3H] ketanserin which was incubated with and without excess of unlabelled 10 µM ketanserin. Tubes were incubated at 37 °C for 15 minutes and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice cold 50 mM Tris sulphate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The receptor binding parameters were determined using Scatchard analysis.

**Analysis of Gene Expression by Real-Time Polymerase Chain Reaction**

PCR analyses were conducted with gene-specific primers and fluorescently labeled Taqman probe of GABA_β_, 5-HT_2A_, cAMP response element-binding protein (CREB), NF-κB, TNF-α, Akt-1 and superoxide dismutase (SOD) which were designed by Applied Biosystems. Endogenous control, β-actin, was labeled with a report dye, VIC. RNA was isolated from the cerebral cortex of experimental rats using the Tri-reagent according to the procedure of Chomczynski and Sacchi. Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 µl contained 0.2 µg total RNA, 10× RT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time-PCR assays were performed in 96-well plates in an ABI 7300 Real-time-PCR instrument (Applied Biosystems). The specific primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for assay on demand and endogenous control β-actin and 12.5 µl of Taqman 2× Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNase free water. The following thermal cycling profile was used (40 cycles), 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples (ΔΔCT = CT Target−CT β-actin). It was further normalized with the control (ΔΔCT = ΔΔCT−CT Control). The fold change in expression was then obtained as (2−ΔΔCT) and the graph was plotted using log2(−ΔΔCT).

**SOD Assay in the Cerebral Cortex of Experimental Rats**

The cerebral cortex was homogenized in a buffer containing 10 mM EDTA, 50 mM Tris-HCl, pH 8.2 and centrifuged at 40000 × g for 15 minutes. The protein content of whole supernatant of the total homogenate was estimated. Cytoplasmic SOD was analyzed after the inhibition by SOD of the pyrogallol autoxidation at pH 8.2. Briefly, 1 ml reaction mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris-HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 minutes with or without 250 µg enzyme protein. The inhibition of pyrogallol was linear with the activity of the enzyme present. Fifty percent inhibition (mg protein · min) was considered as one unit of enzyme activity.
**Immunohistochemical Analysis by Confocal Microscope**

The experimental rats were deeply anesthetized and was transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the brain from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 μm cerebral cortex sections were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked for 1 hour with PBS containing 5% normal goat serum and 0.1% triton X-100. The primary antibodies of GABA<sub>B</sub> (Chemicon, 1:500 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) and 5-HT<sub>2A</sub> (Chemicon, 1:1000 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the brain slices were rinsed with PBS and then incubated with fluorescent labelled secondary antibody (Alexa Fluor 594, code-A11012, Invitrogen) prepared in PBS with 5% normal goat serum and 0.1% triton X-100 at 1, 1000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure was validated by negative controls (data not shown) to ensure that the labelling method accurately identifies the antibody bound to the specific receptors in the cerebral cortex. Expression of GABA<sub>B</sub> and 5-HT<sub>2A</sub> receptors was analysed using pixel intensity method. The given pixel value is the net value which is deducted from the negative control pixel value. All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

**Statistical Analysis**

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a, San Diego, USA). Student Newman-Keuls test was used to compare different groups after ANOVA. Linear regression Scatchard plots were made using SIGMA PLOT (Ver. 2.03). Relative Quantification Software was used for analyzing Real-Time PCR results.

**RESULTS**

**GABA<sub>B</sub> and 5-HT<sub>2A</sub> Receptor Analysis in the Cerebral Cortex of Experimental Rats**

*B<sub>max</sub>* represents the number of receptors and *K<sub>d</sub>* denotes the affinity of receptors towards the ligand. *B<sub>max</sub>* of GABA<sub>B</sub> receptors in GCNP and GSCNP showed a significant decrease (*p < 0.05*) and SCNP showed no significant change with respect to PHNT. In GCNP there was no significant change in 5-HT<sub>2A</sub> receptor *B<sub>max</sub>* when compared to PHNT. The *B<sub>max</sub>* of 5-HT<sub>2A</sub> receptor showed a significant decrease (*p < 0.05*) in SCNP and GSCNP when compared to PHNT. *K<sub>d</sub>* value of the receptors in GCNP and GSCNP showed a significant decrease (*p < 0.01*) when compared to PHNT (Tables I, II).

**Real Time PCR Analysis of GABA<sub>B</sub>, 5-HT<sub>2A</sub>, CREB, NF-κB, TNF-α, Akt-1 and SOD mRNA in the Experimental Rats**

Gene expression studies of GABA<sub>B</sub> receptor in the cerebral cortex was significantly decreased in PHNT (*p < 0.01*), GCNP and SCNP (*p < 0.001*) when compared to C. While considering the receptor gene expression in SCNP and GCNP, there was a significant decrease (*p < 0.01*) when compared to PHNT. The receptor gene expression was significantly decreased in GSCNP when compared to PHNT (*p < 0.001*) and both GCNP and SCNP (*p < 0.01*). The 5-HT<sub>2A</sub> receptor gene expression in SCNP and GSCNP showed a significant decrease (*p < 0.01*) and GCNP showed no significant change when compared to PHNT (Fig. 1). There was a significant decrease in the expression of CREB in GCNP and SCNP (*p < 0.01*) and GSCNP (*p < 0.001*) when compared to PHNT (Fig. 2). The gene expression of NF-κB, TNF-α and Akt-1 showed a significant decrease (*p < 0.05*) (Table II).

**Table I. GABA<sub>B</sub> receptor analysis in the cerebral cortex of experimental rats.**

<table>
<thead>
<tr>
<th>Groups</th>
<th><em>B</em>&lt;sub&gt;max&lt;/sub&gt; (fmol/mg protein)</th>
<th><em>K</em>&lt;sub&gt;d&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>129.30 ± 8.66</td>
<td>68.9 ± 5.80</td>
</tr>
<tr>
<td>PHNT</td>
<td>98.72 ± 6.20*</td>
<td>74.42 ± 4.32*</td>
</tr>
<tr>
<td>GCNP</td>
<td>76.02 ± 5.40*</td>
<td>61.24 ± 4.10*</td>
</tr>
<tr>
<td>SCNP</td>
<td>96.75 ± 5.66*</td>
<td>79.96 ± 3.52*</td>
</tr>
<tr>
<td>GSCNP</td>
<td>74.68 ± 5.32*</td>
<td>60.02 ± 4.09*</td>
</tr>
</tbody>
</table>

*Notes: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. *B*<sub>max</sub> represents the number of receptors and *K*<sub>d</sub> represents the affinity of receptors towards the ligand. *p < 0.001* and *p < 0.05* with respect to C. *p < 0.01* and *p < 0.05* with respect to PHNT. Values are mean ± S.E.M of five separate experiments.

**Table II. 5-HT<sub>2A</sub> receptor analysis in the cerebral cortex of experimental rats.**

<table>
<thead>
<tr>
<th>Groups</th>
<th><em>B</em>&lt;sub&gt;max&lt;/sub&gt; (fmol/mg protein)</th>
<th><em>K</em>&lt;sub&gt;d&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>251.50 ± 26.69</td>
<td>39.32 ± 4.90</td>
</tr>
<tr>
<td>PHNT</td>
<td>210.72 ± 16.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.45 ± 6.32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GCNP</td>
<td>211.32 ± 15.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.81 ± 4.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCNP</td>
<td>174.75 ± 10.69&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>45.26 ± 3.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSCNP</td>
<td>177.68 ± 10.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34.12 ± 4.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Notes: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment. *B*<sub>max</sub> represents the number of receptors and *K*<sub>d</sub> represents the affinity of receptors towards the ligand. *p < 0.001*, *p < 0.05* with respect to C. *p < 0.01* and *p < 0.05* with respect to PHNT. Values are mean ± S.E.M of five separate experiments.
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Increased Cortical Neuronal Survival During Liver Injury: Effect of GABA and 5-HT Chitosan Nanoparticles

**Figure 1.** Real time PCR amplification of GABA\(_A\) and 5-HT\(_{2A}\) mRNA in the cerebral cortex of experimental rats. 
*Notes:* Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. \(^a p < 0.001\) and \(^b p < 0.01\) with respect to C. \(^* p < 0.01\) with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.

The gene expression of neuronal survival factors were increased significantly (\(p < 0.001\)) in GCNP and SCNP when compared to PHNT. The gene expression of neuronal survival factors were increased significantly (\(p < 0.001\)) in GCNP and SCNP when compared to PHNT (Fig. 1).

The level of antioxidant enzyme SOD and its activity were elevated when the ROS content in the cell was increased. The free radical generation was a symbol of apoptosis and increased expression of SOD directly indicated the reduction in ROS. A significant increase (\(p < 0.01\)) in GSCNP when compared to PHNT (Fig. 3). The level of antioxidant enzyme SOD and its activity were elevated when the ROS content in the cell was increased. The free radical generation was a symbol of apoptosis and increased expression of SOD directly indicated the reduction in ROS. A significant increase (\(p < 0.01\)) in GCNP and SCNP when compared to PHNT. The gene expression of neuronal survival factors were increased significantly (\(p < 0.001\)) in GCNP and SCNP when compared to PHNT (Fig. 3).

**Figure 2.** Real time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats. 
*Notes:* Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. \(^a p < 0.001\) and \(^b p < 0.01\) with respect to C. \(^* p < 0.01\) with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.

The level of antioxidant enzyme SOD and its activity were elevated when the ROS content in the cell was increased. The free radical generation was a symbol of apoptosis and increased expression of SOD directly indicated the reduction in ROS. A significant increase (\(p < 0.05\)) in SOD gene expression was observed in GCNP, SCNP and GSCNP when compared to PHNT (Fig. 4).

**Figure 3.** Real time PCR amplification of NF-κB, TNF-α and Akt-1 mRNA in the cerebral cortex of experimental rats. 
*Notes:* Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. \(^a p < 0.001\), and \(^b p < 0.01\), \(^c p < 0.05\) with respect to C. \(^* p < 0.001\) and \(^* p < 0.01\) with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.

**Figure 4.** Real time PCR amplification of SOD mRNA in the cerebral cortex of experimental rats. 
*Notes:* Values are Mean ± S.E.M. of five separate experiments. Each group consists of five rats. \(^b p < 0.01\) with respect to C. \(^* p < 0.05\) with respect to PHNT. Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle treatment (SCNP) and GABA and 5-HT chitosan nanoparticle (GSCNP) treatment.

**SOD Activity in the Cerebral Cortex of Experimental Rats**

The activity of cytoplasmic SOD was significantly increased in GCNP, SCNP (\(p < 0.01\)) and GSCNP (\(p < 0.001\)) when compared to PHNT (Table III).
Table III. SOD activity in the cerebral cortex of experimental rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD concentration (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>03.76 ± 0.20</td>
</tr>
<tr>
<td>PHNT</td>
<td>23.32 ± 1.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GCNP</td>
<td>42.50 ± 3.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCNP</td>
<td>48.98 ± 3.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSCNP</td>
<td>60.04 ± 4.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle (GSCNP) treatment. <sup>a</sup>p < 0.001 with respect to sham operated control. Values are mean ± S.E.M of five separate experiments.

Table IV. Confocal imaging studies of GABA<sub>B</sub> receptors in the cerebral cortex of experimental groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>43.8 ± 0.18</td>
</tr>
<tr>
<td>PHNT</td>
<td>34.59 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GCNP</td>
<td>26.83 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCNP</td>
<td>33.91 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSCNP</td>
<td>22.18 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle (GSCNP) treatment. <sup>a</sup>p < 0.001 with respect to sham operated control. Values are mean ± S.E.M of five separate experiments.

Table V. Confocal imaging studies of 5-HT<sub>2A</sub> receptors in the cerebral cortex of experimental groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>48.9 ± 0.23</td>
</tr>
<tr>
<td>PHNT</td>
<td>39.5 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GCNP</td>
<td>37.8 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCNP</td>
<td>28.9 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSCNP</td>
<td>21.2 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: Sham operated control (C), Partially hepatectomised group with no treatment (PHNT), GABA chitosan nanoparticle treatment (GCNP), 5-HT chitosan nanoparticle (GSCNP) treatment. <sup>a</sup>p < 0.001 with respect to sham operated control. Values are mean ± S.E.M of five separate experiments.

GABA<sub>B</sub> and 5-HT<sub>2A</sub> Receptors Antibody Staining in the Cerebral Cortex of Control and Experimental Rats Using Confocal Microscope

GABA<sub>B</sub> and 5-HT<sub>2A</sub> receptors staining using receptor specific primary antibody and fluorescent labelled secondary antibody showed a significant change in receptor expression. The mean pixel intensity of fluorescent labelling of GABA<sub>B</sub> receptors was decreased (<sup>p</sup>< 0.001) in GCNP and GSCNP and no change in SCNP when compared to PHNT (Table IV, Fig. 5). The mean pixel intensity of 5-HT<sub>2A</sub> receptor staining was decreased (<sup>p</sup>< 0.001) in SCNP and GSCNP and no change in GCNP when compared to PHNT (Table V, Fig. 6).

Figure 5. Confocal image of GABA<sub>B</sub> receptors in the cerebral cortex of control and experimental rats using immunofluorescent GABA<sub>B</sub> receptor specific primary antibody and Alexa Fluor 594 as secondary antibody.

Notes: The scale bar represents 40 μm. Sham operated control (A), Partially hepatectomised group with no treatment (B), GABA chitosan nanoparticle treatment (C), 5-HT chitosan nanoparticle treatment (D) and GABA and 5-HT chitosan nanoparticle treatment (E).
Figure 6. Confocal image of 5-HT$_{2A}$ receptors in the cerebral cortex of control and experimental rats using immunofluorescent 5-HT$_{2A}$ receptor specific primary antibody and Alexa Fluor 594 as secondary antibody. 

Notes: The scale bar represents 40 μm. Sham operated control (A), Partially hepatectomised group with no treatment (B), GABA chitosan nanoparticle treatment (C), 5-HT chitosan nanoparticle treatment (D) and GABA and 5-HT chitosan nanoparticle treatment (E).

DISCUSSION

A great deal of focus has been directed to chitosan nanoparticles to improve drug bioavailability, modify pharmacokinetics and/or protect the encapsulated drug. Chitosan is the second-most abundant natural polysaccharide next to cellulose. Fewer side effects, solubility, poor bioavailability, specific delivery to site of action with good pharmacological efficiency, absorption at intestine, degradation of drug, slow release and effective therapeutic outcome, are the challenges faced by the present drug delivery systems. Chitosan nanoparticles can very well interact with the negatively charged cell membrane by the amino group and make the polymer one of the best carrier for the active compounds to delivery site.

The relationship between the functional status of liver and that of the brain has been known for centuries. In liver diseases, neurotransmission in the brain is reported to be altered. A spectrum of neuropsychiatric abnormalities in patients with liver dysfunction were observed and was characterized by personality changes, intellectual impairments and a depressed level of consciousness associated with multiple neurotransmitter systems, astrocyte dysfunction and cerebral perfusion. Report also showed that there is an onset of mitochondrial damage in brain due to decreased synthesis of glutathione by damaged liver, which was the major glutathione synthesis site.

The liver has a remarkable capacity to regenerate after cellular damage or tissue removal. Liver regeneration is mostly the result of increased mitosis of hepatocytes. Agonists of GABA can act at the GABA receptor complex, and increased concentrations of the agonists are found in the brain in liver failure. Neurosteroids produced in brain during acute liver failure led to increased GABAergic tone and also, elevated intra-cerebral concentrations of GABA significantly decreased ornithine decarboxylase activity in the liver. This was an index for decreased hepatic proliferation and function. There is also an interesting report suggesting that serotonin can potentially contribute to liver tissue hypoperfusion following hepatic ischemia and reperfusion. Sympathetic innervation is important for liver regeneration. Our previous study showed an increased DNA and protein syntheses, which are the cell division markers, in the regenerating liver of GCNP, SCNP and GSCNP when compared to that of PHNT after 24 hours post hepatectomy. During liver injury ammonia metabolism is disturbed and led to a condition called hyperammonemia. Hyperammonemia has been suggested to induce enhanced cerebral cortex ammonia uptake, subsequent glutamine synthesis and accumulation. The changes in cerebral cortex glutamate and gamma-aminobutyric acid could be related to altered ammonia metabolism. The autonomic regulation of GABA was mediated through GABA$_{A}$ receptors and reduction in the GABA neurotransmission in the brain regions enhanced DNA synthesis in liver by facilitating the sympathetic tone. During active hepatocyte proliferation, the serotonin receptor number was decreased in the cerebral cortex of partially hepatectomised rats. During liver injury and hepatic insufficiency, the aromatic amino acid catabolism was altered. Thus the plasma levels of aromatic amino acid increases and enters the brain. The aromatic amino acid tryptophan enhanced the serotonin synthesis in cerebral cortex, which lead to active serotonin mediated neurotransmission. As hepatic cell recovery progresses, the aromatic
amino acid metabolism also reactivated. Thus the serotonin content gets decreased in cerebral cortex.35 In our results also the 5-HT<sub>2A</sub> receptor expression was down regulated in GCNP, SCNP and further decreased in GSCNP when compared to PHNT that supported the regulation of neurotransmission mediated through GABA and 5-HT receptors during liver regeneration.

The transcription factor CREB is a rapidly responding intracellular effector of neurotransmitter signalling and growth factor signalling in mature neurons.39 CREB was controlling the neuronal survival, in part, by controlling transcription of neuroprotective genes. Reports stated that neurotransmitter receptor activation is directly proportional to the CREB gene expression and its translocation to the nucleus.40 In our study, GABA<sub>A</sub> and 5-HT<sub>2A</sub> receptors mediated signalling through CREB was decreased in PHNT, GCNP, SCNP and further decreased in GSCNP that implied decreased GABA and serotonin neurotransmission, which was a sign of increased cell division in regenerating liver.

NF-κB is expressed in diverse cell types in the nervous systems.41 An involvement of NF-κB in neuronal development demonstrates its activation in neurons in certain regions of the brain during neurogenesis. Inhibition of NF-κB by an inhibitor increases cell death and infarct size following transient ischemic insult in rats, suggesting that NF-κB induces survival signalling in neuronal cells.42 Phosphorylation of NF-κB on serines 32 and 36 by IκB kinase leads to its ubiquitination and degradation by proteosomal enzymes, which allows NF-κB heterodimer to translocate to the nucleus and regulate gene expression. In GCNP, SCNP and GSCNP groups the NF-κB level was increased compared to PHNT, which showed an increased neuronal survival and maintenance. One well studied pathway that led to NF-κB activation was by the involvement of cytokine TNF-α through intracellular signalling molecules TNF receptor associated factors (TRAF2 and TRAF6) and activated NF-κB-inducing kinase (NIK), which phosphorylates the IKKs.43 IKK can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNF-α and platelet-derived growth factor (PDGF)-induced NF-κB activation has to require Akt.44 Also, report showed that NF-κB appears to be a target of the anti-apoptotic Ras/PI (3)K/Akt pathway and the expression and activity of Akt was regulated by NF-κB.45 Thus it was clear that during the damage due to partial hepaectomy, the interleukin activation of NF-κB, TNF-α and Akt-1 were required for the survival of neurons in the cerebral cortex. From our observation, in group GSCNP a better cortical neuronal survival signalling mechanism was observed when compared to PHNT. Neuronal death induced by glutamate accumulation46 can be decreased by the treatment with GABA and 5-HT.47 Thus in our study, the combined effect of GABA and 5-HT treatment in GSCNP group showed an increased expression of neuronal survival factors and resulted in reduced cell death.

Brain is rich in polyunsaturated fatty acids and deficient in oxidative defence mechanisms and hence is at great risk of damage mediated by reactive oxygen species (ROS) resulting in molecular and cellular dysfunction.48 An increase in ROS levels automatically favoured an up regulation of antioxidant enzyme, SOD gene expression and its activity,49 which resulted in subsequent suppression of ROS mediated brain damage. SOD is an essential enzyme for detoxifying superoxide radicals to hydrogen peroxide. Oxidative stress was demonstrated in cerebral cortex of hepatic failure induced hyperammonemic and hepatencephalatic animals.50 From our study, a significant increase in SOD activity and its gene expression in rats treated with GABA and serotonin chitosan nanoparticles individually and in combination implied that oxidative neuronal damage in the rat cerebral cortex was less when compared to PHNT.

The present study discussed and pictorially represented (Fig. 7) the potential of GABA and 5-HT, individually and in combination, encapsulated in chitosan nanoparticles for protecting the brain cortical cells during liver injury. The neuronal protection and survival in the cerebral cortex was enhanced in partially hepatectomised rats treated with a combination of GABA and 5-HT chitosan nanoparticles. Thus in addition to active hepatocyte proliferation our study provided new hopes in minimizing neuronal damage in liver based injuries and diseases by utilising nanosized polymeric carriers with active compounds.

**CONCLUSION**

Hepatic insufficiency due to liver damage led to an imbalance in routine brain and body functions. This work revealed the potential of GABA and 5-HT chitosan nanoparticles as a better cortical neuronal survival signalling mechanism from our observation, in group GSCNP a better cortical neuronal survival signalling mechanism was observed when compared to PHNT. Neuronal death induced by glutamate accumulation can be decreased by the treatment with GABA and 5-HT. Thus in our study, the combined effect of GABA and 5-HT treatment in GSCNP group showed an increased expression of neuronal survival factors and resulted in reduced cell death.
nanoparticle treatment in improving neuronal survival and reducing oxidative stress mediated cortical damage after partial hepatectomy and successive regeneration events in rats. The study can be further extended to evaluate the role of neurotransmitter receptor mediated functional alterations in neuronal protection and damage in other prominent brain regions like corpus striatum, hippocampus and brain stem during active hepatectomy proliferation.

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