Investigating the Effect of Serotonin II and III Receptor Inhibitors on the Chronotropic Changes of the Heart to Isoproterenol in Rat Models of Biliary Cirrhosis

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Abstract- Cirrhotic patients have hyperdynamic circulation and at-rest tachycardia, and agents that activate the sympathetic pathway, such as physical practice and pharmacologic stimulations, compared with the normal population, cannot cause enough increase in heartbeat, a condition known as cirrhotic cardiomyopathy. Concerning the presentation of 5-HT2 & 5-HT3 receptors in rat hearts, we used Ketanserin as a 5-HT2 receptor inhibitor & Tropisetron as a 5-HT3 receptor inhibitor to evaluate chronic therapeutic effects of 5-HT2 & 5-HT3 antagonists on the cardiac chronotropic response of cirrhotic rats to adrenergics. Cirrhosis was induced by surgical ligation of the bile duct in Male Wistar rats, and another group remained sham. A week after bile duct ligation or sham surgery, the subjects were given an intraperitoneal injection of either saline or Tropisetron (2 mg/kg). In other BDL & Sharm groups, the subjects were given an intraperitoneal injection of either saline or Ketanserin (6 mg/kg) every other 3 days in the last 3 weeks. Four weeks after bile duct ligation or sham surgery, the atria were isolated and chronotropic responsiveness to Isoproterenol was assessed using a standard organ bath. Our data showed that chronic treatment with Tropisetron (5-HT3 antagonist) in cirrhotic rats could decrease the cardiac chronotropic response. Chronic treatment with Tropisetron can cause a significant decrease in cardiac chronotropic response to Isoproterenol in healthy and cirrhotic rats, even lower than that in cirrhotic rats (without any special treatment). Chronic treatment with Ketanserin cannot change their impaired chronotropic response to Isoproterenol.

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Introduction

Cirrhosis is chronic damage of the liver parenchyma associated with fibrosis and the appearance of regenerated nodules in the liver tissue. During chronic cirrhosis, satellite cells make collagen, leading to liver shrinkage and fibrosis. The amount of remaining healthy cells also proliferates, making regenerated nodules. Cirrhosis can have various causes, such as viral hepatitis, alcohol, medication, etc. Cholestatic manifestations are a clear appearance of the disease. During the disease, developments such as increased portal vein pressure, enlarged spleen, thrombocytopenia, ascites, and encephalopathy may occur. Also, cirrhosis can be associated with many extrahepatic complications. Cirrhosis represents a histopathological definition and has various clinical manifestations and complications. Regardless of the cause of cirrhosis, the pathological characteristics include the creation of underlying fibrosis, that partially destroys the structure of the liver, and the appearance of regenerative nodules. This problem leads to a decrease in the mass of liver cells, and hence their function, along with changes in the amount of blood flow. In the chronic clinical course of cirrhosis, the activation

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of satellite cells leads to more collagen and other extracellular matrix components. Cirrhotic patients have variable amounts of liver flow. Therefore, a distinction should be made between stable and compensated cirrhosis. Many complications of cirrhosis require treatment. Cirrhosis has relatively similar complications regardless of the cause (1).

In patients with previously stable cirrhosis, decompensation occurs due to different causes such as infection, endotoxemia (for example, due to bacterial overgrowth during constipation), excessive alcohol consumption, drug treatment, bleeding from oesophageal varices, and dehydration. These patients need hospitalization, fluid balance, monitoring of the state of consciousness, proper nutrition, and appropriate drug treatments such as diuretics, antibiotics, laxatives (1). In the clinical course of patients with advanced cirrhosis, there are often important permanent complications that are independent of the cause of the underlying disease. These complications include increased portal vein pressure, splenomegaly, ascites, encephalopathy, spontaneous bacterial peritonitis, hepatorenal syndrome, hepatocellular carcinoma, portopulmonary hypertension, coagulation disorders, bone disease, and blood abnormalities (1). During chronic cirrhosis, cardiac chronotropic response changes. Cirrhotic patients have resting tachycardia and hyperdynamic blood circulation; thereupon, factors that lead to sympathetic activation, such as tilting, physical exercise, and pharmacological stimulation, compared to the normal population, cannot cause a sufficient increase in heart rate, and this is due to disorders known as cirrhotic cardiomyopathy (2). However, the sympathetic response to tilting or exercise is increased when measured by norepinephrine concentrations, indicating that the heart's impaired response at the receptor level, or post-receptor, is the cause of this disorder. For example, in cirrhotic patients, ED25 isoproterenol is three times that of healthy controls (2). Due to the presence of serotonin II and III receptors in the heart, we discuss the chronotropic changes of the heart to adrenergic stimulation. Serotonin directly causes vascular smooth muscle contraction, especially through 5-HT2 receptors (3). In humans, serotonin is a powerful vasoconstrictor (except in skeletal muscles and the heart, in which it causes blood vessels to dilate) (4). At least some part of this vasodilatation resulting from 5-HT requires the presence of vascular endothelial cells. When the vascular endothelium is damaged, coronary arteries constrict. As previously mentioned, serotonin can trigger reflex bradycardia by activating 5-HT3 receptors on the nerve terminals of chemical receptors. A three-phase (triphasic) blood pressure response is often seen following serotonin infusion.

At first, heart rate, cardiac output, and blood pressure decrease, which is caused by chemoreceptor responses. Following this decrease, due to vascular contraction, blood pressure increases. The third stage involves reduced blood pressure again, which is caused by dilation of the blood vessels feeding the skeletal muscles. Pulmonary and renal vessels are especially sensitive to serotonin vasoconstriction properties. Serotonin also causes venoconstriction, together with the resulting increased capillary filling, leading to the flushing observed after the administration of serotonin. Serotonin has little direct chronotropic and inotropic effects on the heart, and so are probably not of clinical importance (3,4). However, long-term elevated blood levels of serotonin (as seen in carcinoid syndrome) are associated with pathological changes in the endocardium leading to endomyocardial fibrosis (4). Serotonin causes platelet aggregation by activating surface 5-HT2 receptors (5).

Given the presence of 5-HT receptors in the heart, the inotropic effect of serotonin correlates with increased calcium entry into cardiomyocytes, which has electrical and mechanical effects (6). In rats, serotonin administration in the isolated heart exerts a positive chronotropic effect through 5-HT3 receptors and a negative chronotropic effect through 5-HT2 receptors in the rat heart (7). Recent evidence shows that serotonergic mechanisms in the cardiovascular system are not sensitive to serotonin in the development of hyperkinetic circulation and the persistence of portal hypertension in rats following cirrhosis. Cirrhosis was proven by histological findings in BDL rat models and bile duct ligation was accompanied with a significant increase in spleen weight and portal hypertension. The results have shown that the chronic administration of serotonin antagonists reduces portal hypertension in rats (8). It has also been seen that these drugs exert their portal antihypertensive action by reducing the resistance in the mesenteric artery and collateral circulation of cirrhotic patients (8). In this study, ketanserin is used as a 5-HT2 inhibitor and tropisetron as a 5-HT3 inhibitor, considering the presence of 5-HT2 and 5-HT3 receptors in the rat heart, in order to investigate the effect of chronic treatment with 5-HT2 and 5-HT3 antagonists on "decreased chronotropic response of the cirrhotic animal's heart to adrenergic stimulation". Increased portal venous pressure enters the right atrium after entering the IVC. Now, given the presence of the SA node in the right atrium and the decrease in the chronotropic function of the heart in cirrhosis, along with the observation that

ketanserin can lower the portal vein pressure, we aim to discover if reducing the portal vein pressure can improve the function of the SA node.

Materials and Methods

Animals

Wistar albino rats weighing 250-280 grams (provided by the Department of Pharmacology-Tehran University of Medical Sciences) were used in all stages of the experiment. The animals were kept in the animal house of the pharmacology department under a sleep-wake cycle (12 hours of light and 12 hours of darkness) at a temperature of 22° C and had free access to food and water. All animal procedures were in accordance with the guidelines of animal care and the Council Directive of Laboratory Animals, Tehran University of Medical Sciences, Iran.

Procedures

After being weighed, the rats were divided into two control or Sham-operated and experimental or cirrhotic bile duct ligated (BDL) groups. In the BDL group, a midline abdominal incision was made on the first day. The common bile duct was separated from the underlying tissue, closed at two points, and cut in half. In the Sham group, all stages were performed except closing and cutting the common bile duct. Then, the abdomen was closed. It has been well established that the BDL group develops biliary cirrhosis after 21 days (2).

Sham and BDL animals were divided into three groups. The first group, or those treated with ketanserin, were treated with ketanserin by intraperitoneal injection at 6 mg/kg every other three days in the last 21 days (from day 7 to day 28) (9). The second group, or those treated with tropisetron, in the last 21 days (from day 7 to day 28), were treated daily with tropisetron by intraperitoneal injection at the dose of 2 mg/kg (Considering the protective effects of tropisetron on the heart) (10). The third group was subjected to intraperitoneal injection of normal saline in the last 21 days, and the rest of the study continued to the 29th day. On the 29th day after surgery, BDL rats showed apparent symptoms of biliary cirrhosis, including jaundice, dark urine, and ascites. In previous studies, with the pathological examination of the liver tissue and the significant increase in the weight of the spleen, which is associated with the constant increase in portal blood pressure, it has been well documented that after 21 days of closing the common bile duct in rats, liver cirrhosis develops (2). On day 29, the animals were anesthetized intraperitoneally with 100 mg/kg ketamine and 2 mg/kg diazepam in the Sham group and 0.5 mg/kg in the BDL group. After the animals were anesthetized by ketamine and diazepam (previous studies have shown that the combination of ketamine and diazepam has the least effect on cardiovascular parameters compared to other anesthetic drugs), the chest was immediately split open with scissors, and the animal's heart was removed. A small incision is made on the ventricle to drain the blood from the heart and prevent the formation of clots. Then, the heart is placed in the carbogenated physiological salt solution (KCl; 5 and NaCl; 112 and NaHCO3; 25 and KH2PO4; 0.5 and NaH2PO4; 0.5 and MgCl2; 1 and CaCl2; 1.8 and glucose; 10 and EDTA; 0.004 mM) and gently massaged to remove the rest of the blood inside the heart, whereupon the atrium becomes transparent and can be easily identified from the ventricle. The heart is placed in a Petri dish, and the atria are separated from the ventricles using very fine scissors and forceps. After separating the atria, a knot is tied on the auricle of the atria. The atrium is placed inside the organ bath by these knots so that it is fixed to the rod on one side and connected to the isometric transducer on the other side by a thread. Then, a tension of 1 gram of force was applied to the isolated atrium. Under the tension, the spontaneous beats of the atrium were recorded by an isometric transducer (2,11). The signal obtained from the atrial beats was transferred to the PowerLab device, and the analogue signal was converted to a digital signal with a sampling rate of 10 kHz and recorded using the Chart 7 software. The same software indicated the number of atrial beats. The organ bath provides a suitable environment for the atrium to continue beating for hours, so the temperature is kept constant at around 37^{o} C, with provision of oxygen, glucose, and electrolytes. After the atrium is placed in the organ bath, we allowed it to become stable for 30 minutes, and if no signs of arrhythmia were observed during this time, the study continued.

After 30 minutes of stabilization, the response of the isolated atrium to the addition of adrenergic stimulation (isoproterenol) in the concentration range from 10^{-10} to 10^{-5} M was evaluated and recorded cumulatively with a time interval of 2 minutes (2,11).

Analysis

The dose-response curve was plotted to check the response to isoproterenol. After fitting the data to a sigmoid curve, the EC50 and the maximum response to the above pharmacological interactions were calculated. The data were analysed using Prism software version 5 and are shown as Mean±SEM. In the study conducted to

compare all groups, two-way ANOVA was used since there were two independent variables, i.e., cirrhosis versus Sham-Operated and type of treatment. Bon Ferroni's post-test was used for multiple comparisons. In all analyses, a *P* less than 0.05 was considered significant.

Results

Investigating the effects of different concentrations of isoproterenol on heartbeats in the presence and absence of tropisetron and ketanserin in cirrhotic and sham groups

In this experiment, the effect of different concentrations of isoproterenol (10^{-10} to 10^{-5} M) as a non-selective agonist of β receptors was studied on isolated atrial beats in cirrhotic and control groups.

As shown in Figure 1, chronotropic responses to isoproterenol were impaired following chronic closure of the common bile duct and the development of cirrhosis (P<0.001 and $F_{BDL\ VS\ sham}$ =17.5). Maximum and chronotropic response to isoproterenol in the BDL group were lower than in the control group (Figure 1). There is a significant difference between the sham-saline and BDL-saline groups. Also, there is a significant difference between the sham-saline and sham-tropisetron groups. It seems that chronic treatment with tropisetron causes a significant decrease in the chronotropic response of the heart.

As shown in Figure 2, heart response in the BDL-ketanserin group is not different from BDL-saline. The number of samples in groups is 5-6 samples. Two-way ANOVA was used to investigate the effects of treatment on different parameters.

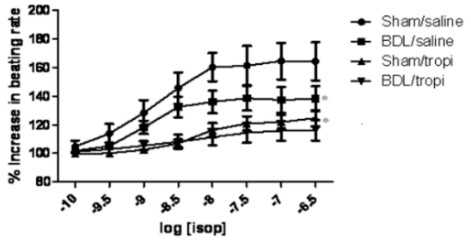

Figure 1. Chronotropic response of the isolated atrium to β-adrenergic stimulation in Sham-operated (Sham) or Cirrhotic (BDL)rat models treated with saline or tropisetron. Chronotropic studies were conducted on spontaneous beating isolated atria. Responsiveness of the isolated atrium to the cumulative concentrations of isoproterenol (from 10-10 to 10 -6.5 M) in sham and cirrhotic animals after receiving tropisetron or saline. Data are shown as mean±SEM. 5-6 samples were used in each group. P<0.0001 was used in comparison with the Sham/Saline group (two-way ANOVA)

*P<0.05 and **P<0.01 compared with the Sham/Saline group at the same concentration (Bonferroni post-test)

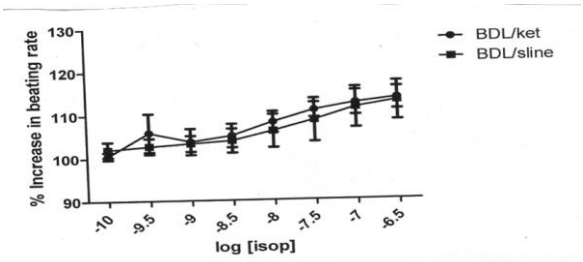

Figure 2. Responsiveness of the isolated atrium to the cumulative concentrations of isoproterenol (from 10-10 to 10-6.5 M) in sham and cirrhotic animals after receiving ketanserin or saline. Chronotropic studies were conducted on spontaneous beating isolated atria. Data have been shown as mean±SEM. 5-6 samples were used in each group. P<0.01 compared with BDL/Saline group

Discussion

It has been well established that in cirrhotic patients, physical and pharmacological stimulations cannot cause the expected increase in the heart rate. This disorder is a part of heart disorders, called cirrhotic cardiomyopathy that is associated with the prolongation of QT interval (defined as the interval between start of Q wave and end of T wave) (12). In cirrhosis, the atrial chronotropic response to beta-adrenergic stimulation is significantly impaired. In the last two decades, many studies have been conducted to understand the mechanism of cirrhotic cardiomyopathy. Although many of the early cases were based on the hypothesis that this issue is due to the down-regulation of beta-adrenergic receptors secondary to increased sympathetic activity, recent studies have shown that it is due to increased nitric oxide synthesis and cardiac protein nitration in cirrhosis. Although there is an impaired chronotropic response to adrenergic stimuli in studies, it is controversial in the presence of inotropic incompetence. Therefore, in this study, we only investigated the chronotropic response. This hypothesis investigated the chronotropic changes of the heart to adrenergic stimulation in cirrhotic rat models after chronic treatment with serotonin 3 and 2 receptor inhibitors and whether the administration of chronic drug treatment with serotonin 3 and 2 receptor inhibitors can improve the chronotropic changes of the heart to adrenergic stimulation in cirrhotic rat models. According to studies, tropisetron of 2-5 mg/kg can exert protective effects on the heart (10).

In this study, tropisetron was used at a dose of 2 mg/kg. We observed that chronic treatment with tropisetron can reduce the chronotropic response of the heart to adrenergic stimulation(isoproterenol) in healthy

and cirrhotic rats. This response is even less than in animals with cirrhosis (without any specific drug treatment) to isoproterenol.

The different functions of serotonin are related to the wide variety of its receptors and effectors. The functional responses of the cardiovascular system regulated by serotonin are very complex due to many receptor subsets and their different activities. From different aspects, serotonin is a neurohormonal substance that plays an important role in cardiovascular regulation. It is well known that serotonin produces positive chronotropic and inotropic effects in the cardiac muscles of different mammalian species. So far, the inotropic effects of serotonin, directly (by stimulating certain serotonin receptors) (13), indirectly (by releasing noradrenaline from sympathetic nerve terminals) (14), or both (15) depending on different species, have been reported.

5-HT4 receptors mediate positive chronotropic and positive inotropic effects in human atrium muscle (16), and 5-HT2A receptors sensitive to ketanserin mediate positive inotropic effects in rat atrium muscle (17). Also, in a study, Mertens *et al*., described that cardiac hypertrophy is related to impaired chronotropic response to serotonin, which is mediated by the 5-HT2 receptor (18). The positive chronotropic effects of serotonin are inhibited by different 5-HT3 receptor antagonists (Tropisetron, Granisetron, Ondansetron, Cisapride, and Zacopride). Still, those 5-HT receptor antagonists that were not specific for the 5-HT3 receptor did not affect this issue. Therefore, the positive chronotropic response to 5-HT is a direct effect and has been suggested to be mediated by the 5-HT3 receptor subtype (19).

In general, 5-hydroxytryptamine, from a concentration of 5 microM to 50 microM, can have a positive chronotropic activity on the isolated rat atrium. Studies show that stimulating the 5-HT2 receptor increases atrial beats effectively. Also, by using a 5-HT2 receptor antagonist (ketanserin), the positive chronotropic effects of 5-HT are lost at a concentration of 5 microM. At higher concentrations (50 microM) of 5-HT, the indirect tyramine-like sympathomimetic effect is the most important mechanism in exerting a positive chronotropic response. As the tyramine-like effect is inhibited by reserpine, the positive chronotropic response of 5-HT is reduced at the concentration of 50 microM but not destroyed. Subsequently, the residual response to 5-HT is destroyed by the 5-HT2 receptor antagonist (ketanserin) (20).

So far, it has been reported that the phosphorylation of p38 mitogen-activated protein kinase (MAPK), which subsequently leads to the induction of heme oxygenase-1 (HO-1), exerts protective effects on intestinal injury (21). Studies have shown that administering a single dose of tropisetron in rats following trauma-hemorrhagic shock exerts protective effects on liver tissue through the activation of p38 MAPK and subsequent HO-1 up-regulation and down-regulation of pro-inflammatory mediators, such as cytokine-induced neutrophil chemoattractant-1 and -3 (*CINC-1* and *CINC-3)*, intercellular adhesion molecule-1 (*ICAM-1)*, interleukin-6 (*IL-6)*, tumor necrosis factor-α *(TNF*-α), and macrophage inflammatory protein-1α *(MIP*-1α) (22). Also, according to another study, a single dose of ondansetron (1 mg/kg) in male rats protects against liver damage after haemorrhagic shock followed by fluid resuscitation, with a similar mechanism through the p38 MAPK pathway (23). According to evidence in rats, cardiotoxicity caused by Doxorubicin occurs with its single dose intraperitoneal injection (15 mg/kg) (10). Some side effects caused by DOX administration are cardiotoxicity in the form of changes in the electrocardiogram and left ventricular dysfunction (24).

Dox causes cardiotoxicity by different mechanisms: DOX leads to an increase in oxidative stress and the release of proinflammatory cytokine, such as interleukin-1 beta *(IL*-$_{1B}$) (25). In a study conducted by Fiebich *et al*., they noticed the inhibitory effect (dose-dependent) of tropisetron on the secretion of *TNF*-$_{\alpha}$ and *IL*-$_{1B}$ (26). Also, DOX leads to the activation of the transcription factor called nuclear factor of activated T-lymphocytes (NFAT), and subsequently, the apoptosis of cardiomyocytes by increasing the intracellular calcium levels, followed by calcineurin activation (27). According to evidence, tropisetron blocks the activation of NFAT signalling cascades (28), and exerts its inhibitory effect by targeting the calcineurin pathway (28,29). Also, DOX causes the loss of cardiac fibers, dilated cardiomyopathy, and impairment of ventricular contractile strength (30). According to another study, administration of tropisetron as a single dose (3 mg/kg; ip) in rats 1 hour before the injection of DOX (15 mg/kg; ip) has protective effects against DOX-induced cardiomyopathy, and in this study, it was observed that tropisetron protects the heart from the negative effects caused by DOX on the contractile strength of the papillary muscle and reduced heart weight (10).

There is evidence that intra-atrial administration of phenylbiguanide causes reflex bradycardia, blood pressure drop, and sympathetic inhibition by stimulating vagal afferent C-fibers, known as Bezold-Jarisch reflex (B-J reflex) (31,32).

In another study, 5-HT (or serotonin) at a dose of 600

and 900 pmol, and chlorophenyl-biguanide (CPBG) at a dose of 10-150 pmol, inhibited the bradycardia (bradycardiac component) from B-J reflex after injection into the nucleus tractus solitarius (NTS). The effect of both agonists was restored by previous local administration of 5-HT3 antagonists (ondansetron 100 pmol, zacopride 100 pmol), but the administration of 5-HT2 receptor antagonist (ketanserin 10 pmol) did not affect this issue. On the contrary, administration of CPBG at a dose of 150 pmol did not affect the inhibition of the B-J reflex derived from lumbar sympathetic nerves. The results showed that stimulation of NTS 5-HT3 receptors leads to the inhibition of the cardiovagal component of the B-J reflex without affecting its sympathetic component. Also, these receptors' stimulation inhibits the baroreflex's cardiac component (32).

The decrease in portal pressure in rats suffering from portal hypertension treated with ketanserin was probably due to a decrease in inflow to the portal venous system secondary to a decrease in cardiac output. In fact, the blockade of 5-HT2 receptors by ketanserin probably led to venous accumulation in the portal system and was followed by reducing venous return to the heart (33). According to another study, ritanserin reduces portal pressure in cirrhotic dogs by reducing intrahepatic resistance (34). However, it is possible that based on the previous study for ritanserin, ketanserin also reduces intrahepatic vascular resistance and portal flow (35). According to studies, serotonin plays an important role in liver regeneration (36). Also, cirrhosis leads to thrombocytopenia and hypersplenism, and splenectomy causes thrombocytosis (37). It is well known that the liver can regenerate and regulate its mass (38). The spleen has an inhibitory effect on liver regeneration (39). Thus, it was shown that splenectomy leads to thrombocytosis, increased liver serotonin levels, a significant increase in phosphorylated extracellular signal-regulated kinases (pERK) and HGF levels, and acceleration of liver regeneration. To investigate whether the increase in serotonin levels after splenectomy can play an important role in liver regeneration, liver cirrhosis was induced by the BDL method. After splenectomy, ketanserin at a dose of (6 mg/kg;ip) was injected every three days for three weeks. Ketanserin did not affect serotonin and HGF levels but significantly decreased pERK protein levels. Therefore, serotonin promotes liver regeneration through a pathway independent of HGF (40).

Our studies showed that inhibition of the serotonin-3 receptor chronically causes a significant decrease in the chronotropic response of the isolated atrium in healthy and cirrhotic rat models to a beta-adrenergic stimulant (isoproterenol). This response is even less than the chronotropic response of cirrhotic animals (not under any special drug treatment) to beta-adrenergic stimulant (isoproterenol). In chronic cirrhotic rats treated with ketanserin, it cannot change the disturbed response of these animals to isoproterenol. In cirrhosis, cardiac responses, including chronotropic responses, are changed, and factors that lead to sympathetic activation, such as pharmacological stimulation, cannot cause a sufficient increase in heart rate compared to the normal population.

Impact Statement

Medicine is a dynamic field of study, and offering new therapeutic considerations in a complicated disease, such as cirrhosis, plays a vital role in advancing human knowledge. Therefore, we were encouraged to plan our study to find new treatments which can improve cardiac chronotropic responses in cirrhotic patients. We showed that chronic treatment with Tropisetron significantly decreases the chronotropic response of the heart in rats with biliary cirrhosis, and it may be possible to improve the chronotropic response of the heart to adrenergics by inhibiting other types of serotonin receptors in cirrhotic patients, and this approach can be applied to better treatment of these patients.

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