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Original Article

Natural Product Goniothalamin Induced NADPH-Diaphorase Expression in the Liver: A Possible Role of Nitric Oxide in Hepatoprotection

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ABSTRACT

Background/Aim: Goniothalamin (GTN) has received significant attention for its selective cytotoxicity toward multiple tumor cell lines, without causing any effect on normal cells. Despite a lack of toxicity, the poor potency of GTN hinders its clinical development. The existing *in vivo* data are also insufficient to support that GTN does not have effect on healthy cells. This prompted us to investigate whether GTN may produce any changes on healthy cells in the organs of mice especially liver as it is vital for metabolism of drugs. The present study aimed to examine the effects of Goniothalamin (GTN), one of the emerging plant-derived anticancer metabolites on the morphology of mouse liver and its possible role in the expression and distribution of Nicotinamide Adenine Dinucleotide Phosphate Diaphorase (NADPH-d), an indirect indicator of Nitric Oxide Synthase (NOS). **Materials and Methods:** Mice were randomly assigned to four main groups (*n*=72): Experimental group (GTN), positive control group (Betulinic acid; BetA), vehicle control group (Dimethyl Sulfoxide; DMSO) and control group (without vehicle). They were further classified into three sub-groups as per the treatment period like 4-day, 8-day and 12-day. **Results:** The NADPH-d expression indicates the presence of NOS, which is an enzyme that involves in the formation of Nitric Oxide (NO). Our results revealed that GTN treatment induced NADPH-d activity in the liver with no significant morphological changes. Furthermore, the expression of NADPH-d significantly increased in the GTN-treated group, when compared to other groups. The NADPH-d positive portal triads, sinusoids and nerve fiber like structures may suggest the role of NO in regulating the blood flow and maintaining the function of liver cells. The distribution of NO in the portal tract suggests the role of NO in transporting bile and the NO detected in hepatocytes may indicate either normal development or to some extent of injury in the hepatic cells. The total surface area stained on the entire surface of the liver lobule was calculated using FIJI software and the results were analyzed by GraphPad software. The stained areas in the test group did not show significant difference when compared to the stained areas in control group. **Conclusion:** NADPH-d expression in the liver of mice suggests that NO signaling may play a key role in GTN-induced hepatoprotection. These results are of direct clinical importance and may pave the way for further development of GTN as a potential pharmaceutical candidate.

Keywords: NADPH-d, Nitric oxide, Goniothalamin, Betulinic acid, Mice, Liver.

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INTRODUCTION

For centuries nature has been the constant resource for various medicinal products.[1-4] Numerous therapeutic plants have been widely accepted and in practice[5,6] and transmitted through generations to alleviate many animal and human ailments including cancer.[6-9] These products recently gained worldwide attention, the reason may be for their fewer side effects[10,11] and rising cost of orthodox medicine.[12-14] According to World health Organization (WHO) estimation nearly 70-80% of the global population currently relies on traditional- medicine[15,16] for various purposes. The advent of new and novel methodologies allows us to decipher ample number of pharmaceutical drug leads from these resources.[17-20]

Chemotherapy is one of the therapeutic options offered for cancer treatment, however, the known side effects and toxicity weakens its effectiveness.[17,21] Nearly 60% of currently engaged anticancer

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compounds are derived from various natural resources[18,22] which include microbial organisms[23] and medicinal plants.[8,24,25] Most of these nature derived compounds are either synthetic or semisynthetic derivative.[26-28]

Plant derived pharmaceuticals like vinblastine, vincristine, taxes and camptothecins[29-31] are highly employed. However, most of this therapeutics as well as numerous pipeline drug leads exhibit potent cytotoxic effects, yet still they are in use in the clinic with care.[25,32] Despite the above fact, nature derived compounds constantly attract criticism mainly due to insufficient toxicity data.[25,33-36] For such reasons, the toxicity of plant derived drug leads need thorough exploration specifically their medicinal properties.[34] Hence, toxicity investigations are vital in determining the toxicity of many therapeutic compounds using appropriate animal.[37-39]

In this regard, GTN, a styryl lactone (R, E)-6-styryl-5, 6-dihydro-2H-pyran-2-one (Figure 1A) compound found in members of a tropical tree Goniothalamus (Figure 1B) recently gained much attention.[40,41] GTN molecule isolated from Goniothalamus macrophyllus and the bark of Cryptocarya caloneura.[42,43] While there are 160 species spread across Southeastern Asia, very few species like Goniothalamus macrophyllus have been recognized for their medicinal properties.[43,44]

TN has been well studied by many investigators for its various actions[45,46] including the induction of apoptosis and anti-inflammation[45,47] along with anti-proliferative properties against diverse cancer cell lines[48-54] when compared to other anticancer agents (e.g. doxorubicin or taxol), however, GTN spares or shown no or less toxic to normal cells such as Chang liver cells and Madin-Darby Bovine Kidney (MDBK) normal kidney cells.[33,44,55-59] GTN has also exerted its anti-proliferative activity against numerous solid tumors, however no toxic evidence was exhibited when single or repeated doses was given.[34,35] Besides, several other extensive investigations also reported that GTN has many more biological functions besides anticancer activions.[40,60] The available data on GTN toxic activity against normal cells, tissues and organs are very limited or insufficient and less convincing, moreover, its specific molecular mechanisms also remain unclear.[40,61] Since limited or no *in vivo* studies are offered to support its effect on normal architecture of various organs. It is imperative to study the effect of GTN on the normal cells and normal organs upon GTN treatment to establish its toxic profile as a safety measure.

Besides, recent efforts on protecting the normal cells from the side effects of chemotherapy have gained enormous attention to harness cell cycle checkpoints.[62,63] Most of cell-cycle specific drugs target cancer cells while they are dividing and few damage cells at the point of splitting, whereas some targets the cells and copies of all their genes before they split.[64,65] However, normal cells contact inhibition with neighboring cells protect these cells from cell death induced by anti-cancer agents which include GTN and BetA.[40,59,66,67] Although GTN molecular mechanisms remain unclear, we consider that the same protective mechanism may be played in this study. Nitric Oxide (NO) is a free radical and ubiquitous signaling molecule present in immune and endocrine tissues, among others and a well-known mediator in numerous therapeutic and immunomodulatory functions.[68-70] Moreover, NO act as a regulatory molecule in many organs, hence, it is crucial to study the impact of GTN on the morphology and phenotypical changes if any and to see NOS/NADPH-d expression pattern in normal organ like liver.[68-70,72-74]

Hence, this investigation was intent to examine the effects of GTN on the expression of NADPH-d activity and its distribution pattern in the mice liver with the following specific objectives: (a). to examine the effect of GTN on the morphology and phenotypical changes if any in the liver of mice due to GTN compound treatment using standard histochemistry. (b). to observe the expression of NADPH-d activity and its distribution pattern in the liver using NADPH-d histochemistry. (c). to analyze the stained areas indicated by the NADPH-d staining within the same groups and general comparison between four main groups by Kruskal-Wallis by Dunn's multiple comparison tests.

MATERIALS AND METHODS

Experimental Design

Seventy-Two (n=72) female BALB/c outbred mice (2-3 weeks old, 23±2 g) procured from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM)\ National University of Malaysia was used. The experiments were carried out according to the procedures adopted by.[68-70] Animals were kept in plastic cages at the laboratory temperature of 22±1°C under controlled lighting with 12 hr light and 12 hr dark. All the cages were placed in the well-ventilated animal laboratory 2 UKM, Kualalumpur. Each cage contained five mice. The cages were labeled accordingly and laid with wood shaving purchased from the Laboratory Animal Resource Unit. Each mouse was able to get enough food and water supply as the pellet and water will be provided once daily. The cleanliness of the cages was concerned by changing the bedding every three days. All the animals received proper human care in accordance with the guidelines prepared by the Animal Ethic Committee of UKM. The approved code to conduct this research was FF/2022/ALI/20-MAY/685-JUN E-2022-JAN.-2023.

Mice were randomized into four experimental groups, which consisted of the normal control, the vehicle control (DMSO), the positive control (BetA) and the treatment (GTN) group. Each of these groups was further subdivided into three smaller groups, which were 4-day, 8-day and 12-day groups with 6 animals in each sub-group. Animals in the control group received no chemical reagent so that they could act as a standard comparative group. For the vehicle control group, all the 18 mice were injected with

5% DMSO solution by intraperitoneal i.p route. Positive control animals received 10 μM BetA solution, whereas the treatment group received 50 μM GTN solution like the vehicle and positive control groups. All these injections were carried out at once on the first day of the experiment. During the injection, the mouse was restrained manually. The needle was not injected too deeply inside the mice to prevent the needle from penetrating the organs around the areas such as small intestine, large intestine and other organs. The mice which have been administered with the solution i.p were marked using a permanent marker pen to prevent double dosing or missed dose. Six mice from the control group along with six each from the vehicle control, positive control and treated groups on 4th, 8th and 12th day after treatment was sacrificed.

Animal sacrifice and dissection of organs

The animals were sacrificed and dissected on day 4, day 8 and day 12. Chloral hydrate (10%; i.p.) was administered to anesthetize the animals. Anesthetized mice were fixed using fine iron nails onto a polystyrene board. A scissor with sharp tips was used to cut open the part near the throat of the mouse. The skin of each mouse was carefully cut to avoid the accidental cutting of blood vessels. Then, the liver was taken out carefully by cutting the ligaments that join the liver to the surface below the diaphragm and to the front part of the abdominal wall. After the required organs were taken out, they were washed with 0.1 M Phosphate Buffer Solution (PBS) to rinse the blood on the organs. Then, the organs were preserved by 4% paraformaldehyde in different vials for different mice dissected in different days. The vials were then stored at 2°C to 8°C. The preserved tissues were subjected to routine histopathological investigations.[71] Five-micron (μM) paraffin sections were stained with Hematoxylin and eosin stain (H and E) and then examined under microscopic and photographed.

NADPH-diaphorase histochemistry

NADPH-d technique described by[72] in the detection of NADPH-d staining was applied. Before staining, the sections were washed using 0.1M PBS by gently dipping the slides inside the solution. Then, the slides with sections were incubated for approximately 30 min to 1 hr in the Tris (Hydroxymethyl) aminomethane (TRIS) buffer (Sigma-Aldrich, US) containing 0.5% Triton X (Sigma-Aldrich, US), 0.2 mM nitro blue tetrazolium; NBT (Sigma-Aldrich, US), 15 mM calcium chloride (Sigma-Aldrich, US) and 1.5 mM β-NADPH (Sigma-Aldrich, US). After that, the slides were rinsed with TRIS buffer solution to prevent further reaction and washed twice with PBS. Then, each slide was dipped into alcohol with water mixture to dry up the slides so that clearer images can be obtained. After drying with different percentages of alcohol, the slides were mounted with a few drops of glycerol and the sections covered with coverslip for microscopic observation.

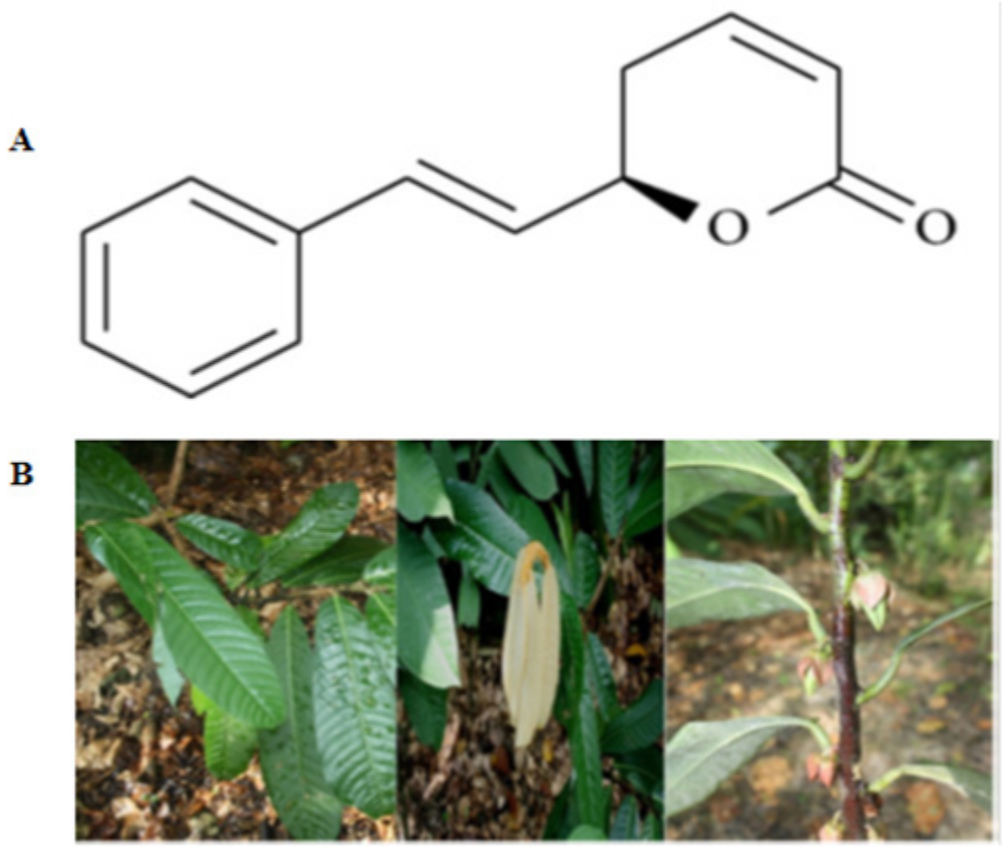

Figure 1A and B: A. Chemical structure of Goniothalamin. B. The image of the plant Goniothalamus macrophyllus (Blume) Hook. Plant images/photos taken by one of the author (Hui Man Yap[1]).

Microscopic observation and analysis

All the gelatinized slides with liver sections were observed under the light microscope with attached camera. The slides were observed under the magnification of x4 only since the magnification larger than that generated blurred images. The stained parts with formazan reaction product were chosen as the images to be captured and will be analyzed using FIJI software and GraphPad software, USA. The stained areas labeled by NADPH-d were calculated by using FIJI software. The stained parts and the unstained parts must be determined carefully as it will affect the interpretation of the data. The total stained areas for the selective liver sections were calculated for each group.

Statistical analysis

Statistical analysis of the NADPH-d-stained areas of the liver sections was analyzed using Kruskal-Wallis by Dunn's multiple comparison tests for non-parametric data using GraphPad Prism version 5 (GraphPad software, USA). The stained areas indicated by the NADPH-d staining were compared for three different days within the same groups, three different days between four main groups and general comparison between four main groups. All measurements were performed in duplicate or triplicate and repeated at least three times. The level of statistical significance was set at less than 0.05 ($p<0.05$).

RESULTS

Histopathological Evaluation

As shown in Figure 2A and B, microscopic examination of the liver tissue of all animals such as control, DMSO, BetA and GTN-treated animal showed that liver obtained from these animals were found normal. The histological and cellular structure of liver from all groups showed unnoticeable differences. In liver, the cellular structure of hepatocytes, sinusoids and central vein of DMSO and BetA groups were like those in normal control group. Similarly, no changes were observed in the liver of mice following the GTN administration compared to normal control and other treated groups. The overall staining pattern of the liver sections of all groups including standard normal control, vehicle control, BetA and GTN groups have shown no difference in staining intensity interpreted by GraphPad software (Figure 2C) and overall total surface areas stained with H and E staining were similar between all four groups.

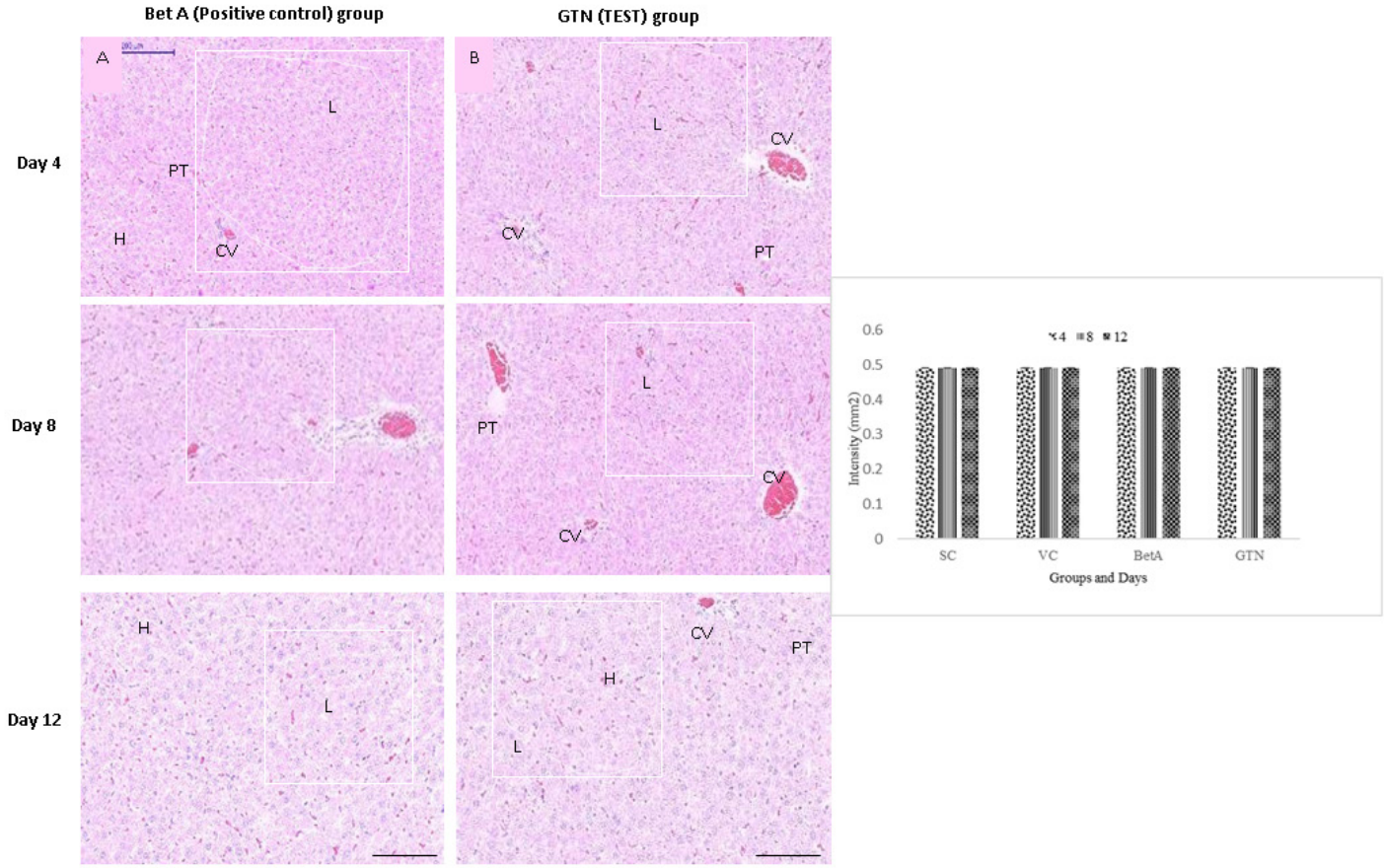

Figure 2: Architecture, various regions and cell types of liver. (A). GTN-treated test and (B). BetA-treated positive control). (C). The intensity (OD) of the HandE staining was quantified using Olympus DP SOFT Software. Data expressed as means±standard deviation of mice (n=6 per group). GTN intensity was compared against control groups (NC) vs. (VC) vs. positive (BetA) groups. p values for NC, VC, BetA and GTN=$p<0.05$). Quantitative results of stained area using HandE in the liver sections have shown no difference in the intensity within as well as between the groups.

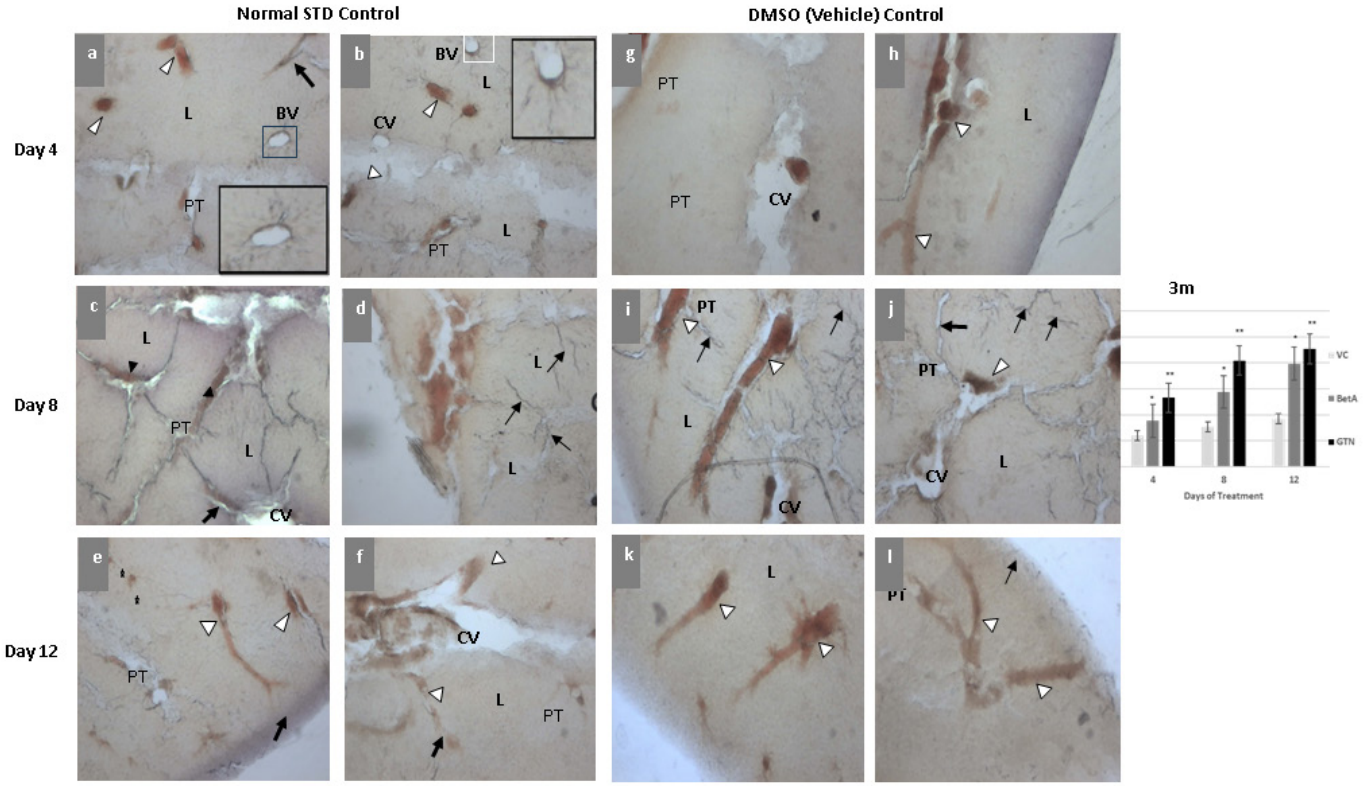

Figure 3: Representative photomicrographs showing liver sections of normal standard and vehicle control (DMSO) group on days 4 (3. a, b, g, h), 8 (3. c, d, I, j) and 12 (3. e, f, k, l) days. Inset outlined in the figure represents the black lined box area of NADPH-d-stained Blood Vessels (BV), medium white arrowhead points the hepatocytes and small thick arrow pointing the NADPH-d perivascular nerve fibre like structures. Letter 'PT' stands for portal triads showing NADPH-d positive staining. Scale bar is 100 µm. The intensity (OD) of the NADPH-d staining (3m) was quantified using Olympus DP SOFT Software. Data expressed as means±standard deviation of mice (n=6 per group) **p<0.001 (GTN) compared against control groups (VC) vs. positive (BetA) groups. p values for CON=*p<0.01 and GTN=**p<0.01).

NADPH-diaphorase expression

This study aimed at identifying any morphological/phenotypical changes induced by GTN and investigate NADPH-d expression in the liver. The study results have demonstrated that NADPH-d staining/reactivity was restricted to areas such as hepatocytes, sinusoids, nerve fiber like structures and portal triad which consisted of a branch of portal vein, hepatic artery and bile duct. In this experiment, in all groups the stained structures were more evident, but the staining pattern and intensity variation was observed. In the normal control group, on day four, light to moderate NADPH-d positive labeled portal triads, hepatocytes and nerve fibers in the liver lobule were seen (Figure 3a and b). On day eight, NADPH-d positive staining was mostly seen in the peripheries of lobules, hepatic sinusoids and nerve fibers (Figure 3c and d). The periphery of the lobule may contain the portal vein, hepatic artery, bile duct and others. image1The NADPH-d-stained hepatocytes and portal triads are evident in the periphery of the lobule on day 12 (Figure 3e and f). Similarly, in the vehicle control DMSO group, the portal triads, nerve fibers and hepatocytes were positively stained with the NADPH-d on day four (Figure 3g and h), day eight, moderately stained NADPH-d portal triads, hepatocytes, nerve fibers that spread around the lobule periphery (Figure 3i and j) but on day 12, mostly hepatocytes were stained with NADPH-d (Figure 3k and l). Compared to normal and vehicle controls, both positive (BetA) and test group (GTN) showed strong NADPH-d staining intensity and exhibited similar distribution patterns. In the (BetA) group, as shown in Figure 4a and b, NADPH-d densely stained portal triads, hepatocyte, innervated nerve fibers mostly around the periphery of the lobules and the central vein and hepatocytes were seen on day four and predominantly hepatocytes were stained on day eight (Figure 4c and d) and day 12 (Figure 4e and f). On day four (Figure 4g and h), eight (Figure 4i and j) and day 12 (Figure 4k and l) treatment with GTN have shown NADPH-d positive hepatocytes, blood vessel, sinusoids and portal triads with nerve fibers in the periphery of the lobule. The overall staining pattern of the hepatocyte cells that spread along the surfaces of the liver lobules of both BetA and GTN groups have shown stronger staining interpreted by GraphPad software (Figure 3m and 4m). However, the overall total surface areas labeled with NADPH-d staining intensity were not similar between all four groups.

DISCUSSION

The present study showed positive NADPH-d staining in the liver of non-immunized GTN-treated mice. The cells labeled with NADPH-d indicate the presence of Nitric Oxide (NO).[68,69,73,74]

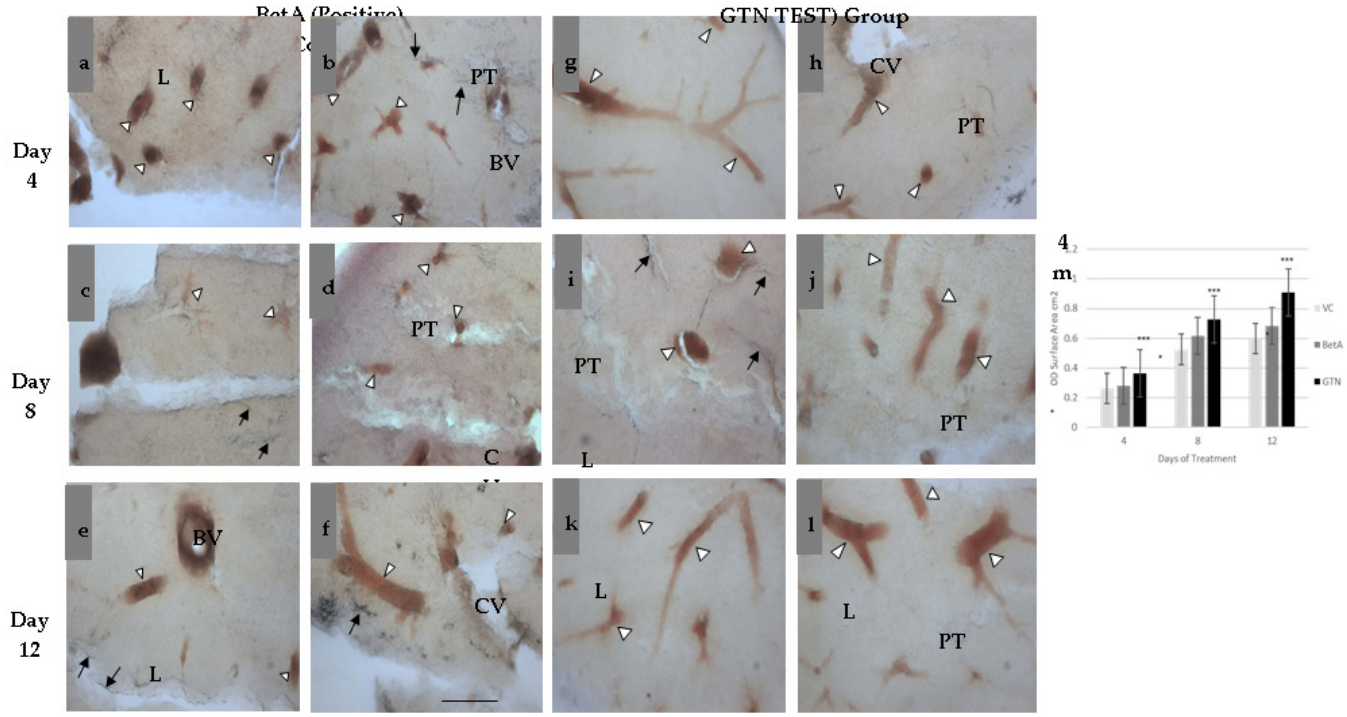

Figure 4: Representative photomicrographs showing liver sections of BetA positive control and test (GTN) group on days 4 (4. a, b, g, h), 8 (4. c, d, I, j) and 12 (4. e, f, k, l). The figure showing the NADPH-d-stained Blood Vessels (BV), Portal Triads (PT) and Central Vein (CV), medium white arrowhead points the hepatocytes and small thick arrow pointing the NADPH-d perivascular nerve fibre like structures. Letter 'L' stands for liver lobule. Scale bar is 100 µm. The intensity (OD) of the NADPH-d staining (4 m) was quantified using Olympus DP SOFT Software. Data expressed as means±standard deviation of mice (n=6 per group) ***p<0.001 (GTN) compared against control groups (VC) vs. positive (BetA) groups. p values for CON=*p<0.01 and GTN=***p<0.01).

However, the expression and distribution pattern NADPH-d is different between the control and test groups throughout the treatment period. Our results found that NO activities were mainly localized in the portal tracts, periphery of the lobule, margins of the central vein, sinusoids, hepatocytes, blood vessel and with some nerve fiber like structures. Based on the structural classification of liver, the two most general functional units in the liver are acinus and lobule.[75,76] Acinus is a smaller functional unit and harder to visualize under the light microscope compared to the lobule.[77,78] Indeed, our results clearly observed lobule staining rather than acinus. Basically, the surface of the liver consists of a lot of hexagonal shaped liver lobules, with portal triads located at each vertex of the hexagonal shaped liver lobule.[79-81] The central structure of a lobule is called central vein and the lobule periphery contains many portal tracts, which arise from the hilum and pass through the liver in a bifurcation fashion. Each portal tract contains portal vein, hepatic artery, bile duct and ductulus, nerve fibers, lymphatic vessels and some inflammatory cells.[75,82-84]

Our results revealed both in the GTN test and BetA positive control group that NADPH-d-stained cellular structures are mostly found in the peripheral region of the lobule, which contains lots of portal tracts.[85] The portal tract contains more than one bile duct, together with a vein and an artery and ductulus, which are responsible in transporting bile.[75,86] The nitrergic activity in the portal tract suggests that NO may play a role in transporting bile.[87-91] To support the above, our study confirmed the presence of NADPH-d positive structures in the portal triads of all different groups, implies that NO may play a key role in bile transportation.

Sinusoids are structures with narrow spaces to carry the blood from the portal tract to the central vein.[75,82] The sinusoidal spaces are entirely lined by the endothelial cells, hepatic-related lymphocytes and Kupffer cells.[77,82] The lymphocytes and Kupffer cells are involved in fighting the infections caused by the bacteria that invade via the body.[92] Our results have found only a few NADPH-d positive sinusoids. Based on the results obtained, the distinct branches on the lobule surface were considered as sinusoids and the light microscopical images may not obviously reveal the entirety of sinusoidal space and its various components.[77] Besides, mild to moderately stained NADPH-d positive hepatocytes were also observed in all four different groups of liver sections that are located entirely on the lobule surface. The hepatocytes are arranged in a 'cord-like' pattern with one to two cells thick and there are sinusoids in between the hepatocytes.[75]

Further, there were some small nerve fibers like structures being stained with the NADPH-d.[63,93] The small nerve fibers were believed to be highly associated with the stellate cells, which are found in the space of Disse, a space found between the endothelial cell lining, the sinusoid and hepatocytes according to Krishna.[75] Based on Esteban *et al*., neuronal NOS (nNOS) can be found in the rat liver,[94] especially in the nerve fibers. From the results obtained, the NADPH-d labeled nerve fibers showed that the nNOS which localized around the entire surface of the liver may involve in blood flow regulation in the sinusoids, vitamin A storage and fibrogenesis.[77,82,95] The innervation of NO-positive perivascular nerves has also been established in many vascular tissues.[96-98] The distribution of supply of nitrergic nerves in the liver are unknown, however, previous studies concentrated on the neural structures contained inside the liver.[99-101] However, the activity of NADPH-d was demonstrated in many parts of the nervous system in mammals.[102-105] The present findings agree with previous results that NADPH-d-labelled cells are present in the rat liver.[73,106,107] Although the current study observed NADPH-d-stained nerve fiber like structures in the perivascular area, NADPH-d neuronal cell bodies were not observed in the liver. The moderate distribution of NO containing nerve fiber like structures moving along blood vessels could reflect a very important role that neuronal NO may play in controlling blood flow in the liver. Both nitrergic positive nerves and vascular endothelium may produce NO to influence blood flow has been described in various hormone producing endocrine tissues like pancreas,[108] thyroid,[74,109] and a series of other tissues including the immune system,[68,102] This observation allows to assume that NO can participate in neurotransmission in the liver.

Previous studies[110,111] have demonstrated that constitutive NOS (cNOS) and inducible NOS (iNOS) can also be detected in the rat liver. The calcium dependent constitutive isoform of NOS is normally present in adrenal gland,[112] brain,[113] pancreas[114] and digestive tract.[115] Besides, endothelial NOS (eNOS) produces NO in the vascular endothelium[116-118] and plays a key role in maintaining the vascular tone.[119] In the liver, eNOS is expressed in hepatic artery, portal vein, central vein, Sinusoidal Endothelial Cells (LSECs) and lymphatic vessels. In addition, stimuli like Vascular Endothelial Growth Factor (VEGF)[120] and stress induces eNOS, which in turn produces small amount of NO. Blood vasculatures are oriented in the portal triads of the liver, NADPH-d staining in this region contributes supports that NO has a role in vascular physiology. Seeing the ability of a blood vessel its dilation requires the activity of eNOS. Therefore, eNOS can most probably be found in the endothelial lining of blood vessels and sinusoids. It plays a role in generating NO to maintain the vascular tone and cellular proliferation of the endothelial cells in the blood vessels and sinusoids.[121] Although, it is well established that NO is recognized as a vasodilator upon its release from endothelial cells[122-124] but various reports[125-127] have shown that NO-mediated vasodilation still occur without involving endothelial cells, where the NO labelled nitrergic nerve fibers may act directly on smooth muscle cells, leading to vasodilation. Neuronal NOS has been involved in such control in the Peripheral Nervous System (PNS).[94,128,129]

Previous investigation by Bandaletova *et al*., described that endotoxin-induced expression of iNOS in the rat liver, which are calcium/calmodulin-independent.[115] Investigators of this study reported that in their result that endotoxin-like substances such as lipopolysaccharide and *Propionibacteriuni acnes* in hepatocytes, Kupffer cells,[130] macrophages in the liver[131,132] and in the neutrophils,[133] eosinophils and lymphocytes in the spleen. There may be a probability that iNOS could be detected in the sinusoidal space as iNOS produced by the Kupffer cells and endothelial cell lining of the sinusoidal space. However, our study did not detect NADPH-d labeled Kupffer cells or endothelial cells from the low magnified images. In this experiment, there may be a chance of iNOS presence in the liver since some of the hepatocytes were labeled with NADPH-d. However, eNOS might be found in the endothelial lining of hepatocytes which may indicate the regulatory role of NO in the hepatocyte such as maintaining the contractility and integrity of the blood vessels.[120] The type of isoform of NOS could not be identified by this experiment as we adopt only NADPH-d histochemistry.

Finally, the results obtained from this study have demonstrated that the comparison between the control and test group may indicate that there was no harmful effect caused by 10 µM BetA and 50 µM GTN on the normal cells. Our results agree with Petsophonsakul *et al*., that GTN can exhibit anti-cancerous activity but not against normal cells. To support this, even 50 µM GTN did not make any considerable phenotypic changes but increased the total surface areas stained by NADPH-d. Likewise, BetA group was compared among day 4, day 8 and day 12, it shows any significant difference among the values between groups. Yet, few limitations still exist and need exploration in future as single dose injection of 50 µM of GTN and 10 µM BetA may be considered as low concentration dose, which may not be enough to cause obvious effect on the normal cells in mice. Therefore, repeated doses of 50 µM of GTN and 10 µM BetA will be considered for future investigations. Furthermore, the period to observe the any changes on normal cells or tissues caused by 50 µM of GTN and 10 µM BetA was quite short i.e., the maximum period of treatment was 12 days only, which may be another limitation to this research.

CONCLUSION

In summary, our findings reveal that both GTN and BetA-treatment induced stronger expression of NADPH-d activity in the liver without producing any significant morphological changes when compared with control groups. However, single dose of either 50µM of GTN or 10µM BetA are considered as low concentration and this may not be enough to induce any significant phenotypic changes in normal organs/tissues. Hence, further investigations

were required with the higher concentrations of GTN and BetA to study the impact of these compounds on normal organs by giving repeated doses instead of single dose. This study's outcome may have direct clinical relevance by providing substantial contribution for drug discovery and development. With our limited knowledge, this may be an important report describing GTN-induced, NADPH-d-mediated NO signaling, which could be of possible molecular mechanism underlying the GTN-induced hepatoprotection.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

NO: Nitric oxide; **NADPH-d:** Nicotinamide adenine dinucleotide phosphate diaphorase; **GTN:** Goniothalamin; **BetA:** Betulinic Acid; **DMSO:** Dimethyl sulfoxide; **WHO:** World health Organization; **MDBK:** Madin-Darby bovine kidney; **UKM:** Universiti Kebangsaan Malaysia; **PBS:** Phosphate Buffer Solution; **TRIS:** Tris (Hydroxymethyl) aminomethane; **NBT:** Nitro Blue Tetrazolium; **β-NADPH:** Beta-Nicotinamide adenine dinucleotide phosphate; **TGF-β:** Transforming growth factor beta; **nNOS:** Neuronal Nitric Oxide Synthase; **iNOS:** Inducible Nitric Oxide Synthase; **eNOS:** Endothelial Nitric Oxide Synthase; **LSECs:** Sinusoidal endothelial cells; **VEGF:** Vascular Endothelial Growth Factor; **PNS:** Peripheral Nervous System.

ETHICAL APPROVAL

The Ethics Committee Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM) confirmed and permitted this study with the approved no. FF/2022/ALI/20-MAY/685-JUN E-2022-JAN.-2023.

SUMMARY

In summary, our findings reveal that both GTN and Bet A-treatment induced stronger expression of NADPH-d activity in the liver without producing any significant morphological changes when compared with control groups. However, single dose of either 50 μM of GTN or 10 μM BetA are considered as low concentration and this may not be enough to induce any significant phenotypic changes in normal organs/tissues. Hence, further investigations were required with the higher concentrations of GTN and BetA to study the impact of these compounds on normal organs by giving repeated doses instead of single dose. This study's outcome may have direct clinical relevance by providing substantial contribution for drug discovery and development. With our limited knowledge, this may be an important report describing GTN-induced, NADPH-d-mediated NO signaling, which could be of possible molecular mechanism underlying the GTN-induced hepatoprotection.

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