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Betulinic Acid: Triterpenoid Derivative Induced NADPH-d Expression in the Urinary System with a Possible Renal Protective Role of Nitric Oxide

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Abstract: The birch tree-derived pentacyclic lupine type-triterpenoid Betulinic acid has demonstrated a variety of biological activities BetA is known for its harmlessness on normal healthy cells. However, recent investigations have indicated that BetA can cause cellular changes in mouse normal embryonic fibroblasts even with a minimal concentration. This report cautioned the use of BetA at the clinical level, which encouraged us to examine whether BetA could produce any key effect on normal healthy cells of any organs in mice. The present study extended its investigation to evaluate whether BetA could induce any changes in the renal system and the expression pattern of NADPH-diaphorase an indirect marker of the enzyme nitric oxide synthase in mice. Our results indicated that BetA exposure induced NADPH-d expression in both organs without causing any significant morphological changes. Moreover, NADPH-d activity patterns in the organs of BetA-treated animals tremendously increased (from day 4 until day 12) when compared to controls. The expression of NADPH-d in both the kidney and bladder implies that NADPH-d-mediated nitric oxide signaling could be a mechanism involved in BetA-induced nephroprotection. These outcomes are of direct clinical importance and could pay the way for the improvement of BetA as an important pharmaceutical product.

Keywords: betulinic acid; BetA; NADPH-d; nitric oxide; NO; kidney; urinary bladder

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1. Introduction

The reactive oxygen species (ROS) play a prominent role in human well-being and are beneficial when combating numerous human diseases [1,2]. The following free radicals such as OH, O_2^- H_2O_2, O_3, HOCI, RO_2, and RO produced during various metabolic activities [3]. Numerous urinary diseases including chronic kidney/renal failure (CRF) have been linked c with redox imbalance, but the mechanism responsible remains unknown [4,5]). It is established that NADPH oxidase is the major source of $O2^-$ ion formation and superoxide dismutase (SOD) participates in the elimination [5].

Nitric oxide (NO) is a ubiquitous gaseous radical species [6,7] present in the immune [8], and urinary system [9] of various species and endocrine tissues [10,11] among others [12–14]. Many studies [15,16] have suggested various contributing factors for chronic renal failure (CRF) like protein nitration, nitric oxide (NO) inactivation, hypertension, and functional NO deficiency. Nitric oxide is a recognized mediator in numerous therapeutic as well as immunomodulatory functions, suggesting its role in immune organs [8,17]. Three different forms of nitric oxide synthases (NOSs) exist they are NOS1, NOS2, and NOS3, which participate in NO and L-citrulline synthesis using cellular oxygen (O_2) from the

L-arginine amino acid, between this NOS1 and NOS3 is constitutive, whereas NOS2 is inducible one [18]. It is known that all three types of NOSs express NADPH-d enzyme activity of NADPH-d [10,12,14,19], suggesting NADPH-d expression pattern is an indirect presence of the enzyme NOS or NO [17,18]. Since the beginning, NADPH-d considered an indicator of NOS [10].

It is well known that NO is the main homeostatic regulator of renal hemodynamics [20,21] and it has been determined as a key player in the pathogenesis of metabolic diseases [22]. To support this, previous studies have established NO deficiency in numerous renal injury experimental models [14,23]. These animal investigations reveal that abnormalities in endogenous NO production are responsible for renal injury, due to the disparity between the production of eNOS and iNOS [24]. Although NOS2 is known to play a considerable role in the urinary system specifically in the kidney, however, its localization in the kidney is not known. However, [25] demonstrated inducible-NOS-positivity in the rat kidneys using immune- and enzyme histochemistry with NADPH-d, further confirmed by RT-PCR. The results have shown the expression not only in the following cell types like interstitial, and glomerular parietal epithelial cells but also in the proximal part of the short-looped and the upper and middle papillary parts of the long-looped descending thin limb. Besides, some inner medullary collecting duct cells and calyceal and papillary epithelial cells also have been shown.

In line with this, numerous pieces of evidence have supported the role of NO in the pathogenesis and cellular changes in diabetic nephropathy (DN), which is facilitated by an increase/decrease in renal NO production and/or its action [14,26]. Despite the above, many studies have raised the question that which isoform of NOS is responsible for NO generation and which type of cells expresses which form of NOS isoform in the DN [27,28]. It is obvious that as of now there is no effective treatment or preventive mechanisms exist for DN to stop its advancement [29]. However, numerous biomedical data indicate that some herbal extracts have beneficial attribution on certain processes with reduced renal function in DM [29,30].

Many renal ailments including cancer need either immune modulation [31] or protection for disease management and care [32] under such circumstances, the host defense system needs activation, which can offer alternates for the existing chemotherapeutics [33]. In this regard, few potential immunomodulatory agents [34,35] have been isolated from several herbs [2,36] or various protective plant-derived natural products (NPs). NPs are small molecules produced either by medicinal plants, microorganisms, or marine sources [30,37,38]. They play a vital role in pharmaceutical drug development as they exhibit a variety of biological properties and deliver different targets throughout the drug discovery process [39]. As chemicals, NPs contain classes of compounds such as terpenoids, amino acids, polyketides, peptides, lipids, proteins, carbohydrates, nucleic acid bases, deoxyribonucleic acid, and ribonucleic acid [40]. Although all NPs have served to inspire intellectual inquiry, one of the most interesting classes of molecules is the terpenes [41,42].

Terpenoids are groups of hydrocarbons that contain terpenes, which include oxygen-containing groups [43,44]. They are usually found in plants and can form cyclic structures such as sterols [8]. Triterpenes compounds were long considered biological inactive phytocompounds until numerous recent investigations have shown their variety of potential biological properties [45]. Normally, pentacyclic triterpenes' dry weight accounts for less than 0.1% of the plant [46]. However, the bark of white birch is one of the exceptions as it contains 34% of triterpene pentacyclic betulin (BE) (Figure 1) [47]. Additional results have further demonstrated that it is possible to obtain about 70 to 90% of active betulin compound [48–50]. They are also present in various other natural sources, which include animal products and microorganisms [51,52].

The ubiquitous presence of triterpenoids in the environment resulted in a variety of medicinal properties [43]. BetA (3b-hydroxy-lup-20(29)-en-28-oic acid) is one of the pentacyclic lupine forms of triterpenoids (Figure 1) and botulin derivatives (betulin oxidation product) [53]. It is widely found in the outer bark of the birch tree (*Betula* spp.) [54,55] but also presents significant amounts of free BetA in the underground parts of the swampy

plant *Menyanthes trifoliata* [56]. For decades, BetA has been employed in various traditional practices [57]. Besides, it can be obtained from other plants source also like *Sarracenia flava* (Sarraceniaceae) [58] trees and shrubs such as *Inga punctata* (Fabaceae), *Diospyros* spp. (Ebenaceae) [59], *Vauquelinia corymbosa* (Rosaceae), *Ziziphus* spp. (Rhamnaceae) [60] and *Syzygium* spp. [41,61].

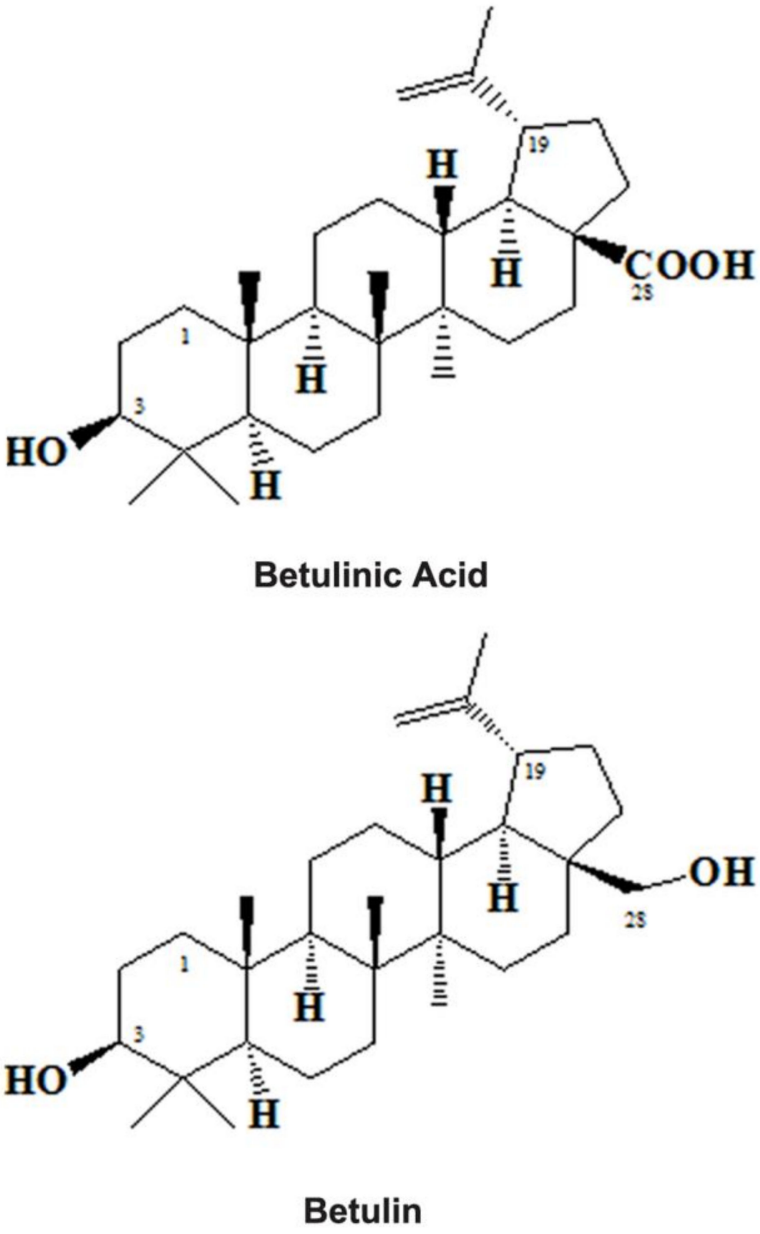

Figure 1. Chemical structures of Betulinic Acid and Betulin.

Many studies have demonstrated that both BE and BetA from various sources displayed their potential through different biological activities such as anticancer [62], anti-inflammatory [63], anti-HIV [64], and anti-bacteria [65]. Besides, it has shown further actions like anti-malarial [66], anthelmintic [67], anti-platelet [68], cardioprotective [69], immunomodulation [8], and so on. It targets various parts of cells like mitochondria [70] and enzyme aminopeptidase N [71], topoisomerase [72], acetyl-coA [73], DGAT [74], NF-kB [75], cell cycle [76,77] and proteasome [78].

In line with this, previous investigations [70,79] have recognized the differential effects or selective toxicity of BetA on cancer cells. These results have been identified as a potential

cancer inhibitor in many in vitro studies using various human metastatic skin cancer cell lines like MEL-1 to 4 [80] and animal models but this compound spared normal healthy cells. This property is unique and different from the other conventional anticancer agents such as vinblastine, taxol, vincristine, etoposide, and camptothecin as they exhibit very toxic and cause damage to both cancer and normal cells [81]. To support further, no major toxic effects of BetA have been witnessed in rodents [70]. Despite the above fact BetA is still considered a weak anticancer agent because its requirements to control cell growth at the in vitro level is μM concentrations, but higher concentrations (2.5 gm/kg/b.wt) are required to inhibit cancer formation in immunodeficient nude mice l [82].

Despite its poor potency and no toxicity on normal cells, still BetA faces challenges in its clinical usage [70] the reason may be that the existing data are not sufficient to support its action on normal healthy cells. When compared to previous reports [70,80] that 10 μM BetA can induce metabolic changes in normal cells without morphological changes [8,82]. The above concerns especially the use of BetA at the clinical level motivated the present study to examine whether BetA could produce any major effect on healthy tissues and cells, more specifically on the urinary organs of mice.

Therefore, the present study designed to determine whether minimal concentrations (10 to 20 μM) of BetA could induce NO production or cause any changes in kidney and urinary bladder cells. NADPH-d normally considered a marker for nitric oxide synthase done by histochemical approach [83]. Thus, in this study, we have investigated the effect of BetA on the NADPH-d or NOS expression pattern of kidneys and urinary bladders of mice by using NADPH-d staining reaction.

2. Results

Based on our observation, in the normal standard and DMSO-treated vehicle control group, mild to moderately stained NADPH-d cellular structures (mean ± SD of 0.1479 ± 0.0408; $p < 0.001$) were observed both in the cortico-medullary parts of the kidney (Figure 2a,d,g). While in the GTN group, the cortical radiate arteries as well as a renal pyramid in the medulla region (mean ± SD of 0.2553 ± 0.0780; $p < 0.001$) showed only moderate NADPH-d expression on day 4 after GTN treatment, that continued day 8th and 12th (Figure 2b,e,h) compared to DMSO control and BetA test group. A similar NADPH-d staining pattern observed in the urinary bladder, however, there is mild or reduced staining in the GTN group, but they showed no cellular changes in the kidney or urinary bladder. This shows that GTN induces a time-dependent fashion of NADPH-d activity in the kidney. Although NADPH-d distribution was like that of control groups at the beginning i.e., on day 4, however, its distribution extends to the entire kidney (both cortex and medulla) for the rest of the treatment periods (i.e., 8th and 12th day of treatment).

A similar but gradual steady increase of NADPH-d expression was evident in the cortical radiate arteries and nephron in the kidney cortex as well as in the medullary pyramids (mean ± SD of 0.3237 ± 0.1340; $p < 0.001$) of the BetA group (Figure 2c,f,i). The NADPH-d activity increased along with the BetA treatment period, which means longer the BetA exposure, the intensity of NADPH-d expression found to increase (Figure 2c,f,i). It is interesting to note that on days 8 (Figure 2f) and 12 (Figure 2i) of BetA treated kidney showed strong NADPH-d staining, however that occurred only in the peripheral cortical radiate arteries (arrow) but not in the medullary arteries as they exhibit only mild staining. It is noteworthy to mention that the cortical area of the kidney, where blood vasculatures are highly oriented, so intense NADPH-d staining in this area (Figure 2i inset) likely to support that NO has a prominent role in vascular physiology [84]. The graphical image in Figure 2j shows the intensity (OD)/staining area (cm^2) of the kidney tissue sections obtained from 4, 8, and 12 days of treatment. In brief, increased NADPH-d activity implies that the effect of BetA on the kidney depends on its exposure time.

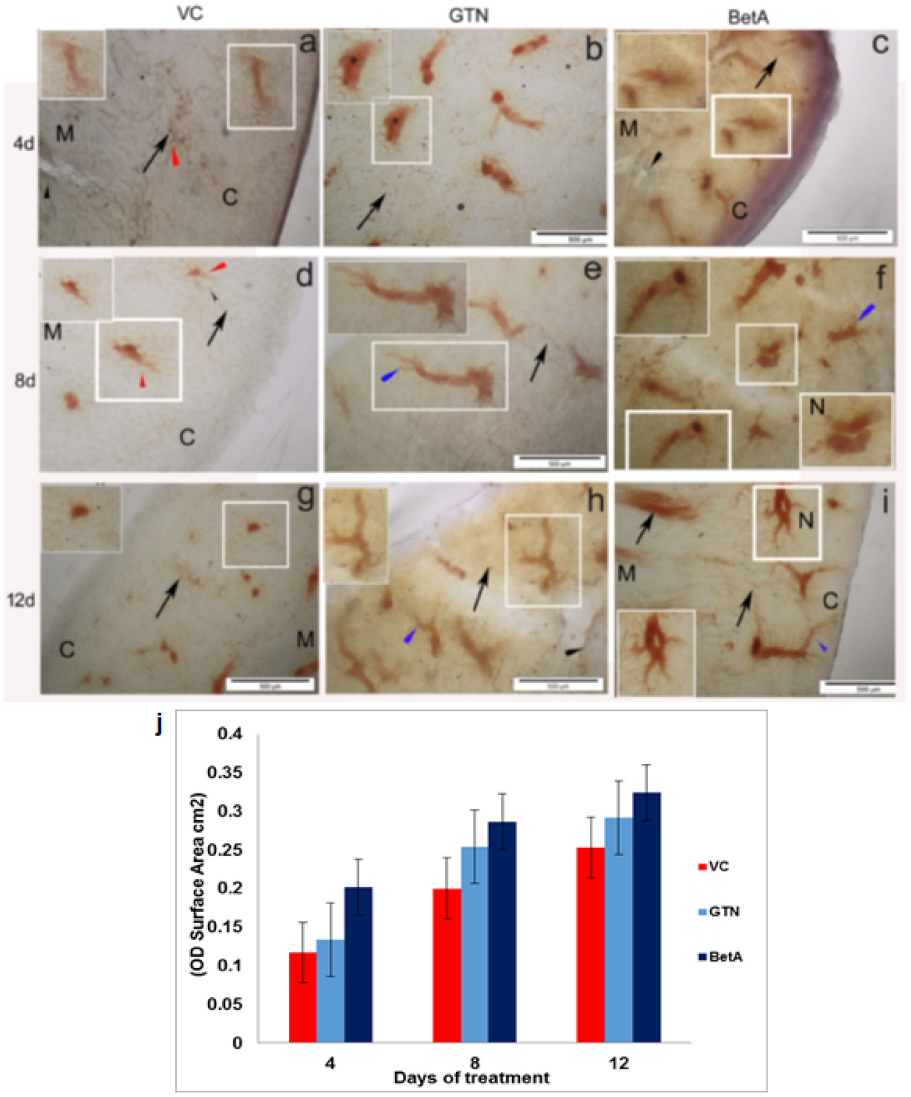

Figure 2. Representative photomicrographs demonstrating Kidney sections of the (**a**–**g**) VC (dimethyl sulfoxide), (**b**–**h**) positive (GTN), and (**c**–**i**) BetA-treated groups on days 4, 8, and 12 after treatment. The inset is representative of the area in the figure outlined in white. Black arrows identify the NADPH-d-stained medullary rays and black arrow indicates blood vessels, the red arrowhead points to the perivascular nerve fibers and the blue arrowhead demonstrates the NADPH-d positive radiate arteries in the cortex (denoted by the letter C) and medulla (denoted by the letter M). The letter 'N' indicates the nephron. Scale bar, 100 µm. (**j**) The intensity (OD) of the NADPH-d staining was quantified using Olympus Soft Imaging cell Sens software version 1.6. Data expressed as means ± standard deviation of mice (n = 6 per group) $p < 0.001$ vs. the VC group, $p < 0.001$ vs. the GTN group. $p < 0.01$ vs. the VC group. GTN, Goniothalamin; BetA, Betulinic acid; NADPH-d, nicotinamide adenine dinucleotide phosphate diaphorase; VC, vehicle control; OD, optical density.

3. Urinary Bladder

Interestingly, a similar NADPH-d expression pattern was observed in the urinary bladder. Both negative and DMSO-treated vehicle control group animals sacrificed on days 4, 8, and 12 have shown NADPH-d staining in various cellular structures. Structures include neuron-like bodies and blood vessels mostly in the outer larger areas of the bladder; however, the intensity of the staining found extremely low (mean ± SD of 0.0827 ± 0.0035; $p < 0.001$) (Figure 3a,d,g). The positive-control (GTN) group on day 4 showed less to mild staining (Figure 3b) but from day 8th and 12th after treatment, NADPH-d activity continuously increased (Figure 3e,h). This shows that GTN induces NADPH-d

in the urinary bladder in a time-dependent fashion like that of the kidney (mean ± SD of 0.1164 ± 0.1120; $p < 0.001$).

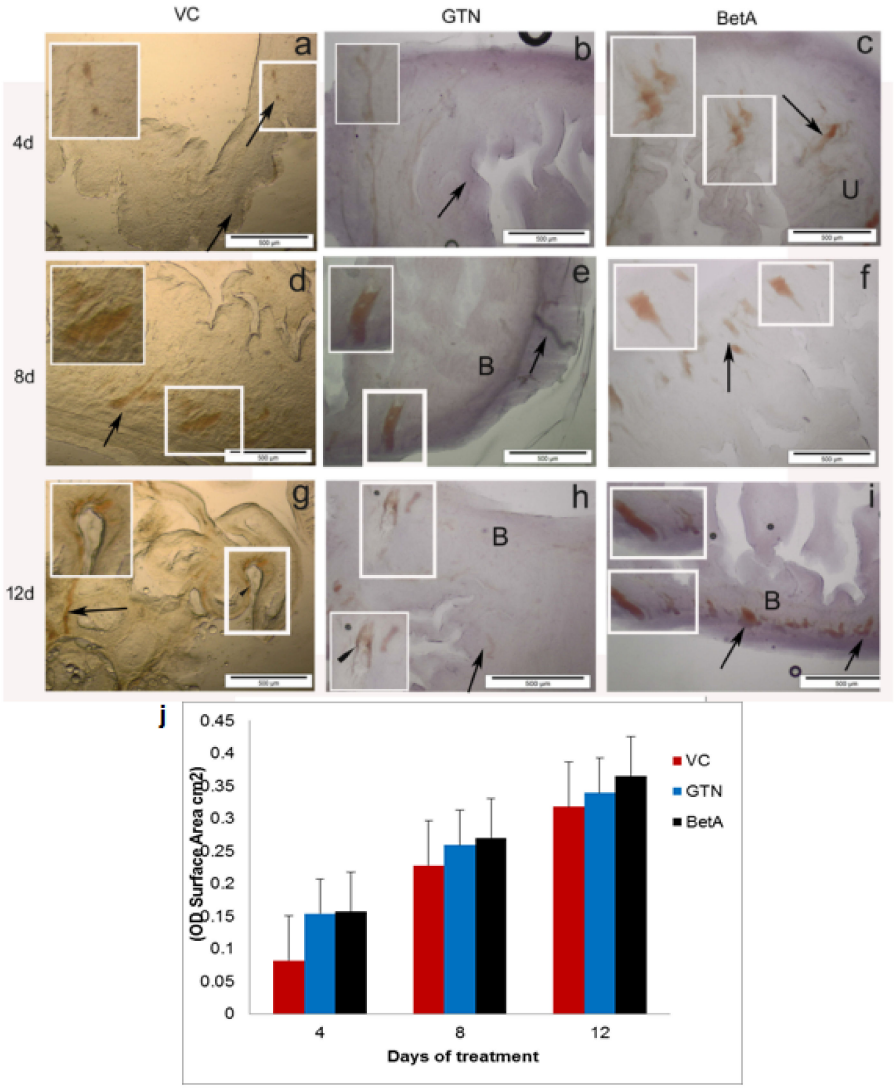

Figure 3. Representative photomicrographs demonstrating urinary bladder sections of the (**a**–**g**) VC (dimethyl sulfoxide), (**b**–**h**) positive (GTN), and (**c**–**i**) BetA-treated groups on days 4, 8, and 12 after treatment. Inset (IS) is representative of the area in the figure outlined in white. Black arrows identify the NADPH-d-stained neuron-like cell bodies and black arrowhead indicate NADPH-d positive blood vessels lining. 'B' indicates the bladder's outer region. 'U' indicates the bladder's inner region. Scale bar, 100 µm. (**j**) The intensity (OD) of the NADPH-d staining was quantified using Olympus Soft Imaging cell Sens software version 1.6. Data expressed as means ± standard deviation of mice ($n = 6$ per group) $p < 0.001$ vs. the VC group, $p < 0.001$ vs. the GTN group, $p < 0.01$ vs. the VC group. GTN, Goniothalamin; BetA, Betulinic acid; NADPH-d, nicotinamide adenine dinucleotide phosphate diaphorase; VC, vehicle control; OD, optical density.

A steady increase of NADPH-d expression and distribution witnessed in every area of the urinary bladder with the higher intensity ($p < 0.001$) in the test group because its expression increased together with the BetA exposure (Figure 3c,f,i). Like the kidney, the bladder also displayed mild to modest NADPH-d staining 4 days following the treatment (Figure 3c) throughout the bladder but from days 8 (Figure 3f) and 12 (Figure 3i) onwards. BetA-treated bladder showed moderate to stronger NADPH-d staining (mean ± SD of 0.2488 ± 0.1220; $p < 0.001$). Neither did we find any substantial structural changes in the positive nor in the test agent-treated urinary bladder. The graphical image in (Figure 3j)

shows the intensity (OD)/staining area (cm^2) of the kidney tissue sections obtained from 4, 8, and 12 days of treatment. In brief, increased NADPH-d activity implies that the effect of BetA on the kidney depends on its exposure time.

4. Discussion

The current study examined the distribution of NADPH-d expression in the GTN and BetA-treated mice kidney and urinary bladder using NADPH-d histochemistry. The results showed varied expression and diverse distribution of NADPH-d in these organs for the entire study period. There are no phenotypical/structural changes have observed both in the kidney as well as in the urinary bladder, however, NADPH-d expression was abundant in the kidney, but less in the urinary bladder. The cortical region of the kidney expressed stronger NADPH-d activity than the medulla region. Although the expression of NADPH-d has seen only after 4 days of BetA treatment, however, significant activity witnessed after 8 and 12 days, suggesting NO production occurred throughout the complete study period. A similar but constant increase of NADPH-d activity also found in the urinary bladder, starting from day 4 may require an interactive outcome of NO in the vascular and other regions of the kidney suggesting it may play a protective role for the nephron [9,85,86]. Although the role of NO in the kidney and urinary bladder not justified, the results of this study corroborated that there is an association between the nephroprotective properties attributed to BetA. The prime effect of BetA-mediated NO engages in the regulation of renal organs [87–89] and our results support the statement in the occurrence of NO demonstrated in the renal system.

Moreover, the present study's histochemical approach revealed the strongest expression for NADPH-d in the renal capsules (comprised of macula densa cells), whereas vascular endothelial showed weaker expression. Our results suggest that renal capsules may produce a substantial quantity of NO. It is probable that in NO-produced target cells, it stimulates soluble guanylate cyclase to generate cGMP from GTP [90,91] and it established that NO/cGMP acts as a vaso-relaxant in the afferent branch of the artery [92]. Previous investigations have also revealed the key outcome of NOS in the efferent branch artery [93]. The obvious discrepancy in inhibition [94] and stimulation [95,96] raises concern about the impact of NO on renin production from granular cells. Since no or limited evidence is available on the potential influence of cGMP on the extra-glomerular mesangial cells, the present results hypothesize and support that NO produced from renal capsules diffuses across the avascular space of the extraglomerular mesangium and may impact the vascular tone of the afferent and/or the efferent arteriole. In addition, NO may enhance renin secretions from granular cells [96].

Since nitric oxide is a gaseous messenger molecule, which has a role in various vital functions including the communication between cells, involved in signaling for vasodilation, neurotransmission, and platelet aggregation inhibition. Physiologically, a constitutive, calcium-dependent isoform of NOS enzymes such as neuronal and endothelial NOS produces a meager quantity of NO for short period, whereas, the inducible isoforms of NOS, which is calcium-independent and require de novo protein synthesis, and produce more amount of NO [97–99].

In addition, NO can be found both in the peripheral as well as in the CNS [100] and in other systems like the endocrine [101,102], immune [103], and renal systems [104,105]. NO exhibits numerous physiological roles in the kidney, which include the regulation of renal and glomerular hemodynamics [106] and natriuretic pressure [107,108], and regulation of medullary perfusion [109]. Furthermore, blunting of tubule-glomerular feedback (TGF) [110], inhibition of tubular sodium reabsorption [111], and involvement in renal sympathetic neural activity modulation also involved [108]. Besides the above, a significant deficiency in NO production in response to increased dietary salt intake has been implicated in the pathogenesis of hypertension [112]. Additionally, NO also plays a significant role in tissue injury as a mediator and portrayed NO as a main r causative factor for end-stage renal failure inflammation [113].

Previous studies have also established that NO deficiency is responsible for the formation and development of chronic kidney disease (CKD) and cardiovascular diseases (CVDs) [114]. Since NO acts locally, the exact NO deficiency location dictates the nephron pathology of renal disease, mediated by multiple molecular mechanisms [115,116]. In agreement with this, previous results have demonstrated that the inhibition of induced chronic NOS in vivo experiments caused various complications such as systemic, glomerular hypertension and ischemia, glomerulosclerosis, tubule-interstitial injury, and proteinuria [116,117]. To support the key role of NO deficiency in renal disease formation, various experimental models have also demonstrated that NO depletion by administration of nitrite (NO2) or nitrate (NO3) delays kidney disease development and decreases blood pressure [89,118]. In addition, routine consumption of NO3 from vegetables and fruits may contribute to cardiovascular protection [119].

It is more evident that several chronic kidney diseases (CKDs) require an improved as well as an effective therapeutic option for their management of them [120]. Pentacyclic triterpene is a triterpenoid found in various medicinal plants. Both BE and BetA are birch tree-derived secondary metabolites found in the leaves, stem bark, and fruit peels [121,122]. These NPs are the main important components employed in the oriental as well as in the traditional medicine systems throughout the world [46,123]. These NPs possess and have shown a variety of beneficial attributions against various disease conditions like anticancer [62], anti-inflammatory [63], and anti-microbial including viruses and bacteria [64,65]. Besides, it has shown further actions like anti-malarial [66], anthelmintic [67], anti-platelet [68], cardio-protective [69], immunomodulation [8], and so on. Besides, its additional targets are mitochondria [70] and the enzyme aminopeptidase N [71], topoisomerase [72], acetyl-CoA [73], DGAT [74], NF-kB [75], cell cycle [76] (Chen et al. 2008; Rzeski et al. 2006) and proteasome [78]. The above targets are currently under drug development for pipeline anti-cancer drugs [124]. However, its efficacy against various renal diseases is unknown or not fully explored [125].

On the other hand, previous studies have demonstrated the selective toxicity of BetA on cancer cells [70,79]. Since BetA considered as potential inhibitor for its anti-cancer action on various metastatic skin cancer cell lines (e.g., MEL-1-4) [80] as well as on animal models [126], this compound specifically spares normal healthy cells [70]. This property is unique and different from the other conventional anticancer agents like vinblastine, taxol, vincristine, etoposide, and camptothecin as they exhibit very toxic and cause damage to both cancer and normal cells [124]. To support further, no systemic toxic actions were also observed in rodents [70] using BetA. Despite the above fact BetA is still considered a weak anti-cancer agent as it requires μM concentrations for the inhibition of cell growth, whereas higher concentrations (250 mg/kg b.wt) are required to control skin cancer in an immunodeficient mouse model [82].

Despite its no of toxicity on normal cells, BetA faces hindrances in its clinical applications [70]. The reason may be due to the non-availability of much in vivo data to support its toxic effect on healthy cells. On contrary, previous reports by [82] observed changes in normal cellular metabolism together with morphological changes. A plausible explanation provided by [82] aimed that the reason for the observed morphological changes by BetA in normal cells may be due to its effect on decreasing oxidative capacity, eliciting increased expression of mitochondrial uncoupling proteins 1 and 2 and triggering liver kinase B1-dependent AMPK activation in mouse embryonic fibroblasts. This enzyme activation further led to an increase in glucose uptake and the glycolysis process [127]. Unfortunately, no in vivo or animal data are available to support the above claim. However, the investigation by [8] described that a minimal concentration of BetA can induce NADPH-d expression, without causing any morphological changes. These contrary reports raised concern about the pharmaceutical significance of BetA and encouraged us to examine whether it could produce any impact on urinary structures like the kidney and urinary bladder of mice, especially on the NADPH-d expression.

Recently, NO has been identified as the main mediator of renal hemodynamics and homeostasis [23]. NO deficiency has also been demonstrated in several renal injury animal models, including during cardiopulmonary bypass (CPB) surgery. Evidence from various experimental data proposes that endogenous NO production abnormality linked with renal injury, due to an imbalance in the production of inducible vs endothelial NOS [24]. Amino acid L-arginine-NO signaling is associated with many physiological roles in the kidney and the disruption in this signaling lead to renal injury [20]. Various investigations have revealed that the macula densa of the kidney produces a significant amount of nNOS/NOS1 [95,110,128,129], where NO has been demonstrated as a key player in the TGF response [110]. Besides the above, [130] have reported that the expression of NOS1 in various structures like non-adrenergic, non-cholinergic neurons within the renal arteries of the hilus, arcuate and interlobular arteries, and rarely in the pre-glomerular afferent branches of the artery. To support this, polymerase chain reaction (PCR) on micro-dissected nephron segments has also shown an elevated level of NOS1 RNA in various regions like inner and outer medullary and cortical collecting ducts (IMCD) [131]. Besides the above, NO produced by eNOS/NOS3 has also shown protective nature in renal vasculature. Increased angiotensin II activity is one of the causes of ROS through NADPH-dependent superoxide generation in the damaged kidney [132,133]. In addition, many other oxidases like Xanthine oxidase, cyclooxygenases, and uncoupled NOS are also involved in the generation of oxidative species, however, NADPH oxidase is the most crucial renal oxidase [5].

It is well known that factors like bradykinin (BK), acetylcholine (ACh), and various other endothelium-dependent ones are promoting water and salt loss through the production of cGMP and the formation of NO [134,135]. Previous studies have demonstrated that these factors influence renal blood vessels by resulting in the alteration in glomerular hemodynamics. In the kidney, like choroid plexus and ciliary processes, cGMP alters fluid secretion and stimulates protein phosphorylation in the secretory epithelium [136,137]. In line with this, the present study results also support the possible role of NO and cGMP.

Previous studies have demonstrated that BetA upregulates eNOS but reduces NADPH oxidase induction in human endothelial cells through PKC-independent mechanisms [121,122]. Triterpenoids have the potential to reverse eNOS uncoupling and augment eNOS enzyme activity by phosphorylation of eNOS at serine 1177 and dephosphorylation of eNOS at threonine 495 [138]. However, the effect of BetA on eNOS expression remains unclear [139] so as in the renal organs, hence it needs further investigation.

In line with this, emerging evidence indicates that BetA has shown renal-protective properties [46,70]. Renal fibrosis considered as an end-stage renal failure that progresses from CKD. It is because of the abnormal increase of extracellular matrix (ECM), as a result, it leads to kidney tissue loss and function [140]. However, in the experimental model of CKD, BetA treatment reversed the loss of functions [125] and up-regulate pro-fibrotic proteins like TGF-β, CTGF, hydroxyl proline, type I collagen, and fibronectin. Besides, tubule dilation, degeneration of glomerulus, and vacuolation with the deposition of collagen fibers also attenuated [69,125]. If there is an impact in the renal tissues due to BetA exposure, it predicted that NO might play regulatory roles in these tissues. In addition to the above, BetA exhibits protection against myocardial ischemia-reperfusion injury mouse model by increasing blood flow but reducing oxidative and nitrosative stress [141].

It is well known that NO function as a vasodilator following its release [142,143] from endothelial cells even from the BetA-treated ones [138,144]. The innervation of NO-positive perivascular nerves has been established in numerous vascular tissues [145,146]. The distribution of such neural structures reported also inside the thymus [8,147,148]. Besides, NADPH-d expression also demonstrated in several regions of the mammalian brain [149–151]. The present study results agree with the earlier investigation that NADPH-d-positive cells exist in the mammalian kidney including rats and mice [25,136]. Although the present study witnessed only NADPH-d, positive nerve fibers not neuronal body-like structures in the perivascular area of the kidney. It is interesting to note that the reasonable distribution of NO-positive nerves traveling alongside the blood vessels could reflect the

role of neuronal NO may be controlling blood flow through both the kidney and urinary bladder. Both NO-positive nerves and blood vessel endothelium may produce NO to influence bloodstream has been reported in the neural system [150] and endocrine organs like the pancreas [152], thyroid [11–13], and a series of other organs. These findings allow if NO can participate in neurotransmission in the kidney and urinary bladder. It is interesting to note that apart from blood vessels, NO may also involve in the regulation of regulatory activity of cortical and medullary cells of the kidney by its production in these cells [103]. Thus, BetA may be a promising pharmaceutical or biological response modifier and may reinforce the renal protection of a host. Although the present study employed a simple but most reliable NADPH-diaphorase histochemical method to demonstrate the presence of NO in the renal system, however, authors admit that involving combined NOS indirect immunofluorescence or immunohistochemistry, immunoelectron microscopy techniques along with more advanced techniques like next-generation sequencing or by microarray analysis at the mRNA level could have been adopted to confirm these interesting results obtained in the current study as they may reproduced NO presence and provided more detailed information and new insights to identify more pharmacological targets, which may benefit for future investigations.

5. Materials and Methods

5.1. Experimental Animals and Chemical Requirements

All animal model investigations conducted in this study were under the Universiti Kebangsaan Malaysia (UKM) Animal Ethics Committee (UKMAEC; FF/2020/ALI/20-MAY/685-JUNE-2020) guidelines. Female BALB/c mice (six weeks old) subjected to the study monitored under appropriate conditions. Betulinic acid (Sigma-Aldrich, St. Louis, MO, USA) used as a test (BetA 10 µM) drug, and Goniothalamin (GTN 50 µM) used as a positive control drug obtained from Merck—Life Science, Malaysia, whereas DMSO (Merck, Darmstadt, Germany) (0.05% DMSO) used as vehicle control.

5.2. Animal Treatment and Sample Collection

Animals used in this study broadly divided into four separate groups and each contains 48 animals: (1) test, (2) positive, (3) negative and (4) a normal control groups. The above-categorized mice were further subdivided equally into three subgroups with each having 6 animals following the treatment regimen (4 days, 8 days, and 12 days). The present study followed the protocol described by [12].

5.3. NADPH-Diaphorase Histochemistry and Tissue Morphology Analysis

Cryo-protected tissue sections collected from each group thawed at normal RT for 30 min, washed twice in PBS, and subjected to NADPH-d staining. β-NADPH (Sigma-Aldrich (M) Sdn Bhd, Subang Jaya, Malaysia) used as a substrate with the addition of nitro blue tetrazolium (NBT) (FISHER SCIENTIFIC (M) SDN BHD), a salt that produces an insoluble blue formazan precipitate visible under the light microscope [8,12].

5.4. Statistical Analysis

One-way ANOVA followed by Bonferroni multiple comparison tests (GraphPad PRISM v. 4.0, San Diego, CA, USA) used to perform intensity data analysis. All numerical data expressed as mean ± SEM and the differences considered statistically significant at $p < 0.05$.

6. Conclusions

In summary, our findings indicate that NADPH-d activity correlates with NOS activity. Although the present study employed a simple but most reliable NADPH-diaphorase histochemistry to demonstrate the presence of NO, still it requires NOS indirect immunofluorescence/immunohistochemistry or immunoelectron microscopy and more advanced techniques like next-generation sequencing or by microarray analysis at the mRNA level

to confirm the NO presence. These additional techniques may provide more detailed information and identified new pharmacological targets. However, the present results reveal that BetA treatment induces NADPH-d expression in both the kidney and urinary bladder without producing any substantial morphological changes in these renal structures. The outcome of the present study has direct pharmaceutical importance, which may contribute to the development of new novel drugs to improve the quality of human health and life. Based on the available literature and our understanding of BetA in the renal system, the present results are particularly important as it describes BetA-induced NADPH-d-mediated nitric oxide signaling in the kidney and urinary bladder, which could be the potential molecular mechanism underlying BetA-elicited renal protection in the treated animals.

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Abbreviations

BetA	Betulinic Acid
BE	Betulin
NADPH-d	NADPH-diaphorase
μM	micro-Molar
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
eNOS	endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
OH	Hydroxyl radical
O_2^-	Super Oxide
H_2O_2	Hydrogen Peroxide
O_3	Ozone
HOCI	Hypochlorous acid
RO_2	Alkoxyl radical 2
RO	Alkoxyl radical
CR	Chronic Renal
CRF	Chronic Renal Failure
SOD	Superoxide Dismutase
NOS1	Neuronal nitric oxide synthase
NOS3	Endothelial nitric oxide synthase
NOS2	Inducible nitric oxide synthase
DN	Diabetic nephropathy
NPs	Natural products
HIV	Human Immunodeficiency Virus
DGAT	Diglyceride acyltransferase
NF-kB	Nuclear factor kappa

MEL-1	Human metastatic Skin Cancer cell line 1
UKM	Universiti Kebangsaan Malaysia
UKMAEC	Universiti Kebangsaan Malaysia (UKM) Animal Ethics Committee
GTN	Goniothalamin
DMSO	Dimethyl sulfoxide
ANOVA	Analysis of variance
cGMP	Cyclic guanosine monophosphate
cGTP	Cyclic guanosine diphosphate
CNS	Central Nervous System
TGF	Tubule-glomerular feedback
CKD	Chronic kidney disease
NO_2	Nitrite
NO_3	Nitrate
b. wt.	body weight
AMPK	5′ AMP-activated protein kinas
CPB	Cardiopulmonary bypass
PCR	Polymerase chain reaction
IMCD	Inner medullary cortical collecting ducts
ROS	Reactive Oxygen Species
BK	Bradykinin
Ach	Acetylcholine
PKC	Protein kinase C
ECM	Extracellular matrix
TGF	Transforming growth factor
CTGF	Connective tissue growth factor

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