

## Therapeutic effects of iNOS inhibition against vitiligo in an animal model

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### Abstract

Nitric oxide (NO) is involved in several biological processes, but its role in human melanogenesis and vitiligo need further studies. Previous studies revealed that exposure to UVA and UVB were capable of the inducing nitric oxide production in keratinocytes and melanocytes through the activation of constitutive nitric oxide synthase, whereas inducible nitric oxide synthase overexpression has been reported to play an important role in hyperpigmentary disorders. The aim of this study was to evaluate iNOS inhibitor aminoguanidine (AG) as a therapeutic agent in our mouse model of vitiligo. In this study, male C57BL/6J Ler-vit/vit mice were purchased to evaluate the effect of iNOS inhibitor (aminoguanidine) (50 and 100 mg/kg) and L-arginine (100 mg/kg) in a mouse model of vitiligo induced by monobenzone 40%. Moreover, we used phototherapy device to treat the mice with NBUVB as a gold standard. The findings revealed that monobenzone was capable of inducing depigmentation after 6 weeks. However, aminoguanidine in combination with monobenzone was decrease the effect of monobenzone, while L-arginine play a key role in promoting the effect of monobenzone ( $P < 0.001$ ). Based on the phototherapy, the efficacy of phototherapy significantly increased by adding L-arginine ( $P < 0.05$ ). Taken together, we suggest that iNOS inhibitor can be a novel treatment for the prevention and treatment of vitiligo by combination of NBUVB therapy, furthermore; NO agents like L-arginine could also increase the effectiveness of phototherapy. Taken together, this pilot study showed significant repigmentation of vitiligious lesions treated with iNOS inhibitor plus NBUVB therapy, where other aspect including expression of an inducible iNOS, NO and TNF levels remained to be evaluated in mice model.

**Key Words:** Vitiligo, Nitric oxide, iNOS, Monobenzone, Treatment.

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Vitiligo, a skin disease, is a common progressive depigmentation of epidermis that results in the destruction of melanocytes in the depigmented parts of the skin, mucous membrane and retinal wall. As a result, irregular white patches appear in different areas of the body. Vitiligo has been estimated to be one of the most frequent pigmentary diseases, accounting for about 0.1–2% of the worldwide population.<sup>1-3</sup> The pathophysiology of this disease is not well understood; however, several theories about the pathomechanism of vitiligo have been previously defined, such as genetic, biochemical, immunological, and environmental theories.<sup>4,5</sup> On the other hand, increasing evidence indicated that there has a correlation between vitiligo and other autoimmune disease of the endocrine glands,<sup>6,7</sup> as well as a lymphocytic infiltrate was found to play an important

role in development of disease when detected in perilesional skin of actively spreading vitiligo.<sup>8</sup> Several cell lines has been described to play their role in vitiligo development including lymphocytes, keratinocytes, and endothelial cells, involving in melanocyte migration, proliferation, and differentiation.<sup>2, 9-11</sup> Increasing evidence supports that vitiligo could be a disorder of the entire epidermal melanin unit.<sup>12</sup> Especially, keratinocytes has been revealed to contribute to the melanocyte homeostasis and its changes affecting melanocyte dysfunction in vitiligo epidermis.<sup>1,13,14</sup> Vitiligo has been defined as a multifactorial disease with autocytotoxic, neural, and autoimmune susceptibilities as well as environmental factors.<sup>15,16</sup> Nitric oxide as an intracellular messenger plays an important role in the synthesis of an enzyme called NO synthase, which consists of three

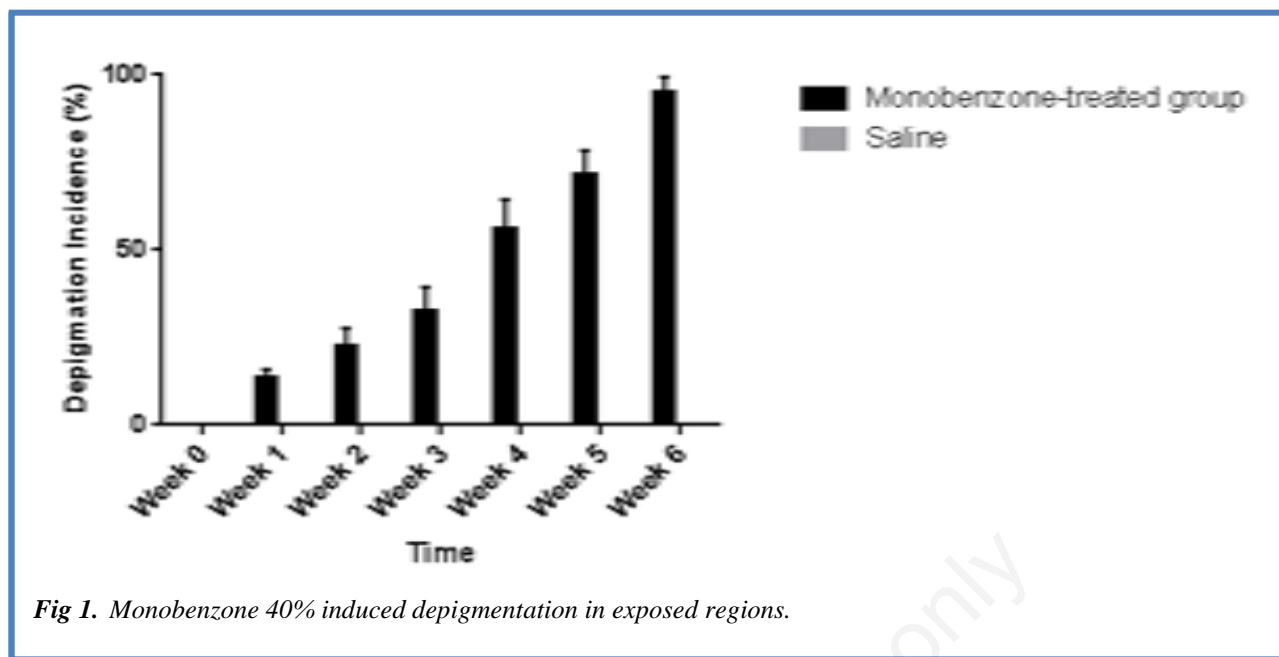


Fig 1. Monobenzene 40% induced depigmentation in exposed regions.

isoforms: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS).<sup>17-30</sup> Nitric oxide has been suggested as a potential factor in inhibiting cell proliferation, differentiation, and apoptosis. This free radical gas can be considered as a great challenge, when a key role plays in pathogenesis of a lot of kind of autoimmune diseases such as vitiligo. Additionally, it is likely to be involved, not only in melanocyte dysfunction and/or destruction, but also in the accumulating toxic intermediates of melanin synthesis and in elevating reactive oxygen species.<sup>17-20,22-27</sup> A growing body of evidence highlights that exposure to UVA and UVB may be capable of producing nitric oxide, especially in keratinocytes and melanocytes, where it is involved, not only in increasing tyrosinase activity via activating constitutive NOS, but also in the synthesis melanin.<sup>21, 31</sup> The use of NBUVB can improve treatment of vitiligo by stimulating the proliferation and migration of melanocytes, as well as stimulating the differentiation of *melanocyte stem cells*. UVA and NBUVB have been introduced as common types of phototherapy in vitiligo. Despite the similar effect of both interventions, narrowband ultraviolet B (NBUVB) phototherapy can be more effective for patients suffering from vitiligo due to the lack of side effects of Psoralen.<sup>32,33</sup> The topical use of immunosuppressive agents, including glucocorticoids and calcineurin inhibitors, have been introduced as the first line treatment, while the use of phototherapy has been considered as the second line for treating these patients.<sup>32</sup> iNOS is involved in the production of NO, where promotor polymorphisms of iNOS gene and NO overproduction has been found to be associated with the pathogenesis of vitiligo, leading to melanocyte destruction.<sup>34-38</sup> Based on above-mentioned data, the current study was aimed to demonstrate the association of vitiligo with iNOS in phototherapy and to investigate

the pathophysiology of this disease using a pharmacological inhibition of the iNOS, where a mouse model of vitiligo was induced by monobenzene.

## Materials and Method

### Ethics Approvals

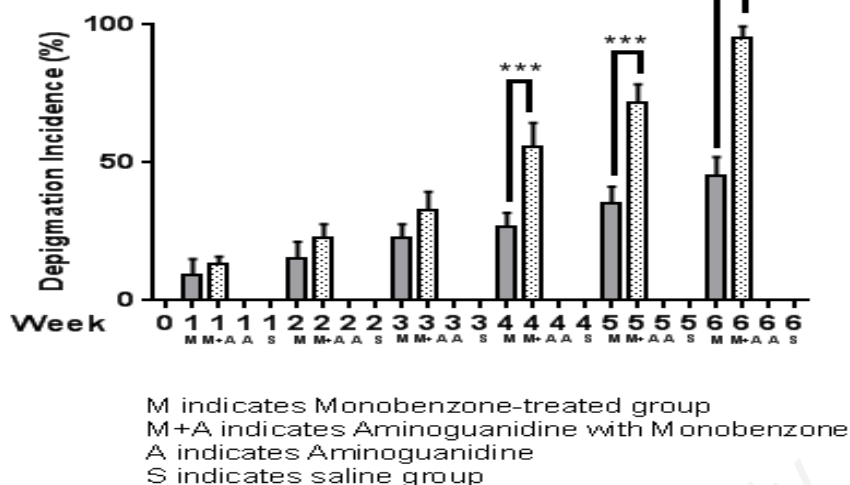
All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) and institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, TUMS). Each experimental group consisted of 6 to 8 animals.

### Animals

In this study, male C57BL/6J Ler-vit/vit mice were obtained from Pasteur Institute, Tehran, Iran (weighing 25-30 g). Animals were then housed in Plexiglas boxes (25 × 25 × 15 cm), under standard conditions (temperature: 22 ± 2 °C, humidity: 50 ± 10%, 12-h light-dark cycle, and free access to food and water).

### Phototherapy device

BIOSKIN®, a phototherapy device is equipped with a short arc generator that provides a beam of visible and ultraviolet radiations, and is also filtered by an interference filter for obtaining UV-B narrow band. The operator is capable of regulating emission time, and plays its role in regulating emission time by accompanying a time-controlled shutter. Furthermore, BIOSKIN® is equipped to prepare a spectrum of intensity up to 400 mW/cm<sup>2</sup>, where provide coverage from 300 to 320 nm for emission spectrum, with an emission peak of 311 nm. Mice were then irradiated three times a week for 4 weeks. The narrow band UV-B (NBUVB) irradiation was performed using a BIOSKIN® device. It is worth noting



**Fig 2.** Effect of aminoguanidine (100 mg/kg) plus monobenzene 40% on depigmentation

that vitiligo patches were next irradiated by excluding mucous membrane and genital part for each micro-phototherapy session. Although BIOSKIN® equipment is able to provide a large spectrum of intensity (0–400 mW/cm<sup>2</sup>), the intensity of 50 mW/cm<sup>2</sup> was employed in the present study for all animals during all sessions. The initial dose of irradiation was 20% less than minimum erythema dose (MED) that was applied to vitiligo area at least 3 days before the beginning of the treatment. During the subsequent sessions, the dose in each session increased to 20% until the development of erythema. Additional dose of the next session was diminished by 20% only in the erythematous area when erythema was developed.

#### Experimental design

In the present study, sex-matched mice were divided in 6 groups of 10 mice. In the first part of this study we employed the vitiligo-inducing agent monobenzene in the mice model, as previously described by Zhu et al.,<sup>39</sup> highlighting that monobenzene-induced vitiligo is a valid mouse model of vitiligo. In the first group (control group), mice received only monobenzene cream 40% on the mouse tail and skin on the back of the mouse and thereafter skin hypo-pigmentations were investigated every week as compared to intact part. In the second group, the mice receive phototherapy (BIOSKIN® device; 50mW/cm<sup>2</sup>) in combination with the monobenzene, which was considered a positive control. In the third group of mice, the role of iNOS in development of vitiligo was then evaluated. Therefore, beginning the first day after vitiligo induction animals were treated with aminoguanidine (AG), an injectable iNOS inhibitor, at daily dose of 50 and 100 mg/kg (i.p. 6x weekly) to clarify the NO pathway. In group 5, the combined effect of this inhibitor with phototherapy was

investigated. In the sixth group, injectable NO inducer was daily used at dose of 100 mg/kg (L-arginine) and the combined effect of this drug was investigated by phototherapy. As a matter of fact, the effects of L-arginine and AG on development of vitiligo were assessed by the vitiligo-inducing compound monobenzene. Optimal treatment dose of aminoguanidine was determined by implementing a dose-response model, assessing increasing doses (50 mg and 100 mg). All drugs were purchased from Sigma, St Louis, MO, USA and afterward dissolved in saline. Animals received drugs intraperitoneally (i.p.), except for monobenzene.

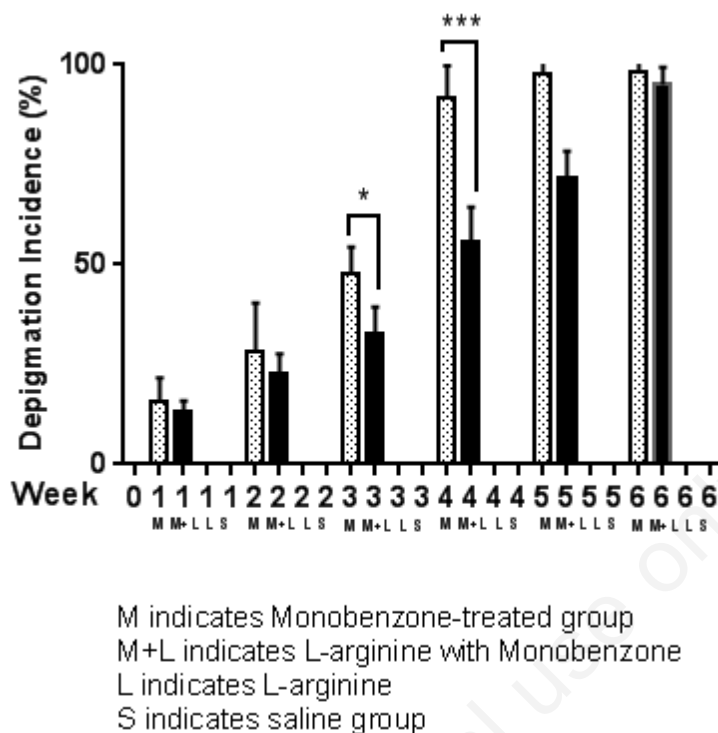
#### Results

##### Monobenzene induced vitiligo

In the first step of this study, we tried to evaluate monobenzene 40%-induced depigmentation from 1 to 6 weeks after exposure. Analysis revealed that monobenzene 40% can induce vitiligo with a mean of 95% depigmentation after 6 weeks, which was associated with the increasing trend of vitiligo. One-way ANOVA analysis revealed that monobenzene could significantly induce depigmentation after 6 weeks ( $F [6, 35] = 218.2$ ,  $P < 0.001$ , Fig1). Post Tukey's analysis showed depigmentation of exposed regions using color cell compression, as given by a computer software after 6 weeks ( $P < 0.001$ ). As shown in figure 1, we suggested an increased trend of depigmentation in initiation phase on exposed area.

##### Co-treatment of monobenzene with iNOS inhibitor

Following mentioned experiment, we provide a co-treatment of iNOS inhibitor (aminoguanidine at 100 mg/kg) with monobenzene 40% to clarify the interaction between nitric system and vitiligo. Statistical analysis



**Fig 3.** Effect of L-arginine (100 mg/kg) plus monobenzene 40% on depigmentation.

revealed that co-treatment of aminoguanidine (100 mg/kg i.p) with monobenzene 40% could significantly decrease vitiligo-inducing effect of monobenzene (F [12, 65] = 127.1; P<0.001; Fig 2). Post hoc analysis demonstrated a significant difference 4 weeks after treatment (P<0.001). In addition, the difference between groups was determined as about 50% of depigmentation after 6 weeks. Overall, aminoguanidine (100 mg/kg) decreased 50% of depigmentation after about 6 weeks.

#### Co-treatment of monobenzene with NO agent

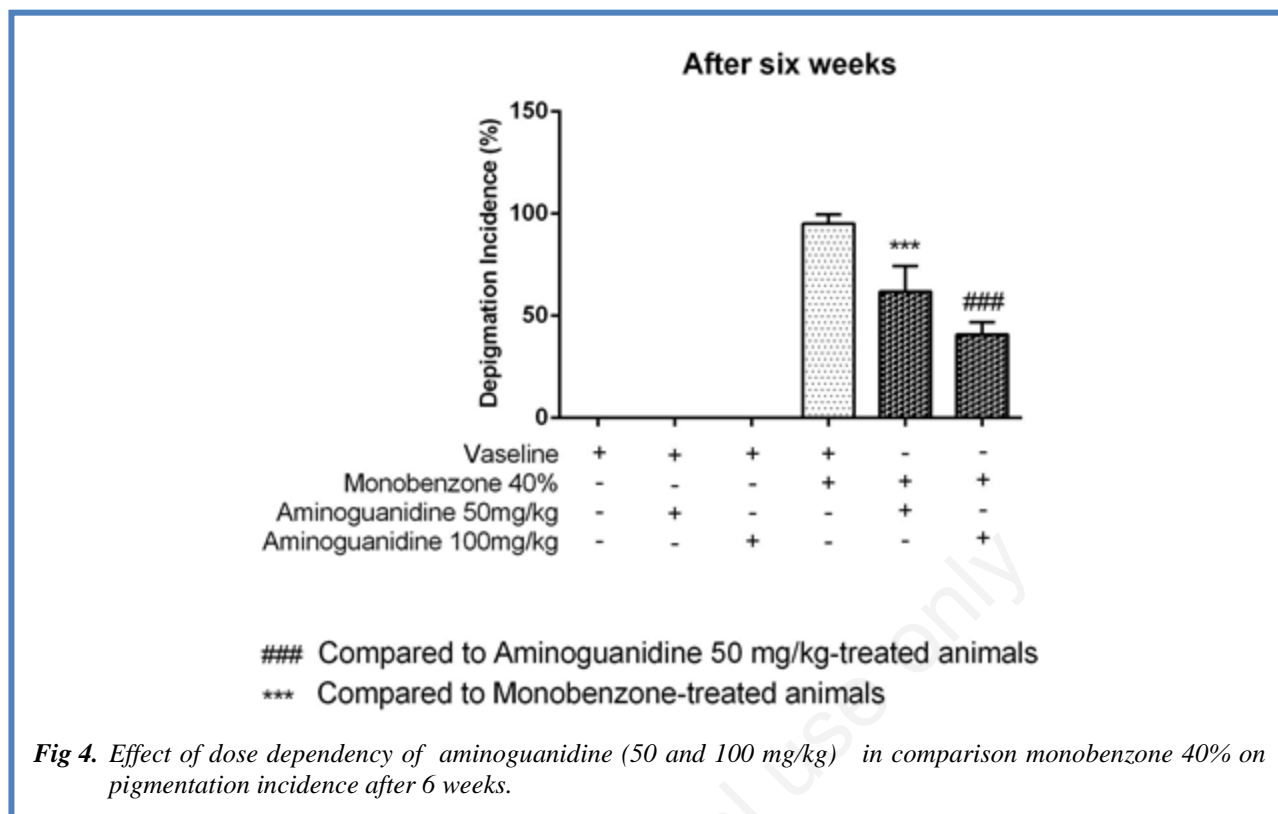
In the next step, we use NOS agent (L-arginine at 100 mg/kg) in combination with monobenzene 40% to evaluate the interaction between increasing NO level and vitiligo (F [12, 65] = 181.2; P<0.001; Fig3). One-way ANOVA analysis demonstrated that a co-treatment of L-arginine (100 mg/kg i.p.) with monobenzene 40% was significantly associated with an increased level of vitiligo-inducing effect of monobenzene. Additionally, post hoc analysis revealed a significant difference between groups after 3 weeks of treatment (P<0.05). In addition, the difference between groups comprised of 35% of depigmentation. Furthermore, a significant difference was only observed after 4 weeks of treatment (P<0.001). Overall, L-arginine (100 mg/kg) significantly accelerated the depigmentation after about 3 weeks (Figure 3).

#### Dose dependency of aminoguanidine on inducing vitiligo

In the next step, we tried to show the dose dependency of aminoguanidine 50 and 100 mg/kg as previously indicated by our experiments. In order to evaluate the dose dependency of iNOS inhibitor, administration of aminoguanidine (50 and 100 mg/kg) with monobenzene 40% were applied in the current study (F [5, 30] = 273.0; P<0.001; Fig 4). One-way ANOVA analysis suggested that administration of aminoguanidine (100 mg/kg) resulted in induction of depigmentation after 6 weeks as compare to aminoguanidine (50 mg/kg), (P<0.001). This experiment showed that aminoguanidine dose dependently could be linked to decreased depigmentation-inducing effect of monobenzene following 6 weeks treatment (Figure 4).

#### Effect of iNOS inhibitor on phototherapy

Finally, we evaluated the phototherapy by using NBUVB as the common treatment of vitiligo in combination with L-arginine to clarify the effect of NO level on NBUVB induced pigmentation (F [2, 15] = 10.12; P<0.001; Fig 5). Analysis revealed that a combination therapy, consisting L-arginine and NBUVB was significantly capable of enhancing the pigmentation of the exposed area, when compared with NBUVB treatment (P<0.01). Duration of NBUVB radiation therapy is about 4 weeks, where a



combination therapy of L-arginine and NBUVB increased the effect of NBUVB (Figure 5).

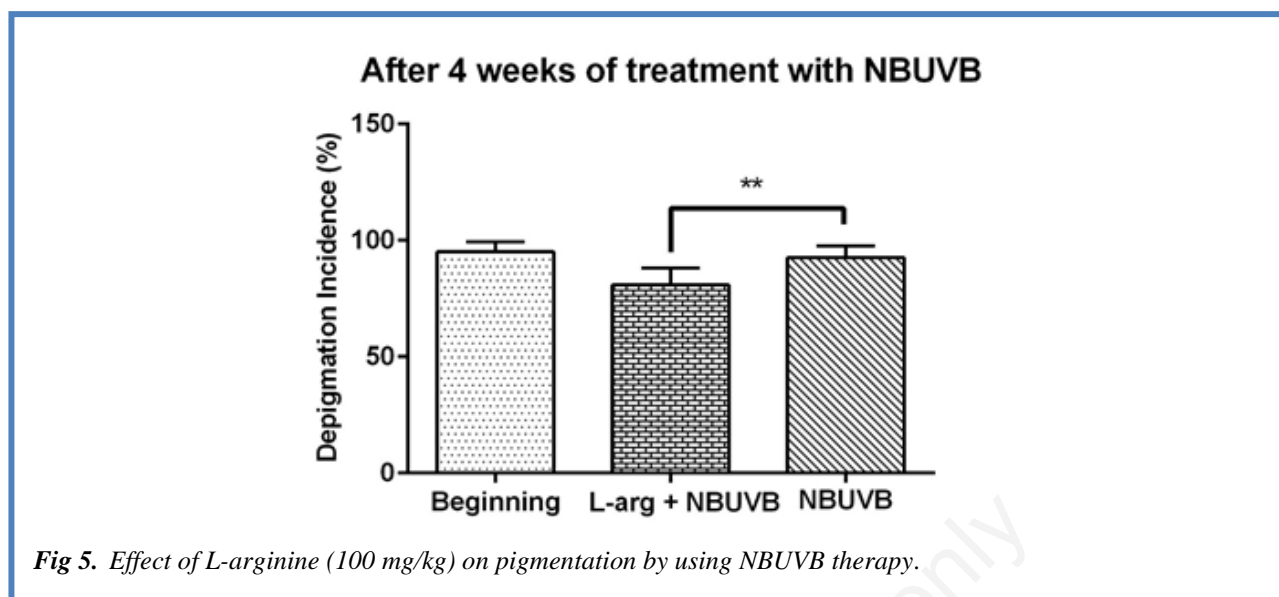
### Discussion

In the current study, we demonstrated that aminoguanidine could inhibit vitiligo-inducing effect of monobenzene after about 4 weeks of treatment. In addition, our results verified that L-arginine could significantly increase the effect of NBUVB treatment in mice model of vitiligo. Nitric oxide has been defined to be a noteworthy mediator that plays a crucial role in numerous biological procedures including melanogenesis, microcirculation, and keratinocyte response to ultraviolet radiation, cell growth and differentiation. Nitric oxide is known to be a major neurotransmitter that plays a key role in the pathophysiology of many diseases of the nervous system, such as epilepsy, schizophrenia, anxiety, and autoimmune disease. Nitric oxide is a chemical messenger that can easily pass through cell membranes, and unlike other neurotransmitters, it does not accumulate in synaptic vesicles and does not release through exocytosis. It seems that nitric oxide plays a crucial role in regulating various body responses, in association with other neurotransmitter systems, such as norepinephrine, serotonin, dopamine, and glutamate. An increasing body of evidence indicates a possible role for NO in hair follicle biology, where is not a well-known regulator in the human hair follicle and the hair-growth cycle.<sup>38,39</sup> NO is known as an important mediator of inflammatory responses in the body. Moreover, its

precarious role in several hyperproliferative and autoimmune disorders has been previously conformed.<sup>40-43</sup> L-arginine is defined to be a biological precursor of NO, a nerve regulator in the brain, by the Nitric Oxide Synthase (NOS) enzyme. The enzymatic family of NOS consists of three isoforms that include inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). All three NOS isoforms play their crucial role in different parts of the brain. Unlike iNOS, nNOS and eNOS are both calcium / calmodulin-dependent, where preclinical evidence supporting important role for this isoforms in cell homeostasis, and alteration of immune and cytotoxic response<sup>31, 44, 45</sup>. It is noteworthy, that eNOS and nNOS were originated in numerous tissues, which have been identified to synthesize by different cell including keratinocytes, melanocytes, and fibroblasts in skin. Ample evidence suggests that expression of iNOS is under the regulation of different cytokines including IL-1, IL-6, IL-23, IL-17, IL-33, IFN- $\gamma$  and TNF- $\alpha$ ,<sup>32,46-49,50-63</sup> which play a key role in inducing the depigmentation. In this regard, our study should be considered in light of a limitation, where further studies are needed to clarify the expression of cytokines such as TNF- $\alpha$  in the future. IFN- $\gamma$  and TNF- $\alpha$  as Th1 cytokines, because increasing evidence supports a role for Th1 response in mediating vitiligo. A study by Taïeb described vitiligo as an inflammatory disease similar to other skin inflammatory diseases, including psoriasis and atopic dermatitis. This research reported that the elimination of inflammation can be considered as a priority in the treatment of this disease.<sup>64</sup>

Vaccaro et al. have shown that UVA and UVB radiation

level under L-arginine treatment. In addition, using



were capable of triggering the synthesis of NO (NOS) enzymes in keratinocytes and melanocytes. They have suggested that an increased level of NO may increase tyrosine kinase activity and then triggered melanin formation, but excessive increased of inos could lead to processes that result in depigmentation. Aforementioned study exhibited that the NO imbalance play a key role in the development of vitiligo, where inhibitors of this factor can potentially play a role in improving this complication, and recommend the assessment of these inhibitors as a priority.<sup>65</sup> Many studies suggested the use of topical corticosteroids as the first line of treatment for vitiligo. Moreover, some studies emphasized on the systemic effectiveness of these drugs for reducing inflammation, and symptoms, as well as inducing repigmentation.<sup>65</sup> On the other hand, immunomodulators (e.g., tacrolimus and calcineurin inhibitors) and reducing the activity of T cells by decreasing the expression of inflammatory cytokines (e.g., IL-2, IL-3, IL-4, IL-5, interferon (IFN), and TNF) along with the granulocyte-macrophage colony-stimulating factor (GM-CSF) can be suggested as effective therapeutic strategies for vitiligo by targeting them that are recently being evaluated.<sup>66-67</sup> Previous studies revealed that the production of high NO level could be associated with the increased activity of iNOS, confer self-destruction of melanocytes, leading to a decrease in De novo attachment of melanocytes at the extracellular matrix,<sup>65, 68-70</sup> where such evidence can follow skin depigmentation.<sup>71</sup> Recent mechanism have also attributed to initial imbalance of epidermal cytokines at the affected areas in promoting tetrahydrobiopterin, iNOS activation, and NO overproduction, resulting in an increase of self-destruction of melanocytes.<sup>65,72,73</sup> In the present study, vitiligo skin was induced by monobenzone 40%, where early findings demonstrated a significant degree of efficacy with appropriate responses, when L-arginine was applied, leading to a decrease in the NO

iNOS inhibitor could decrease the efficacy of monobenzone in the present study. Therefore, emerging evidence has persuaded us to hypothesize that iNOS and NO levels increased in vitiligo lesions compared to healthy controls. In particular, the increased activity of iNOS might be responsible for the overproduction of NO in vitiligo model. Vitiligo is linked to some factors such as increased cytokines expression and iNOS activation that play pivotal roles in the depigmentation process even, if NOS overexpression not being considered as the only important factor involved in vitiligo. An increasing body of evidence indicates that NOS has been overexpressed in vitiligo lesions in comparison with healthy subjects, as well as NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels. The increased activity of iNOS has been also suggested to be potentially linked to the increased level of NO in vitiligo, leading to decrease/death of melanocytes up to depigmentation.<sup>31</sup> Therefore, in the present study, we used an inhibitor to demonstrate whether iNOS inhibition, which is associated with a reduction in NO production, can lead to lesion healing. It is noteworthy that NO increased in vitiligo, as shown preciously by studies.<sup>31</sup> But its types and possible interventions have not been thoroughly investigated as compared to presented findings herein. On the other hand, evidence from in vivo and in vitro studies supports that the melanocytes can be never entirely disappeared in the depigmented epidermis, where these melanocytes are capable of recovering their function in vivo and in vitro hydrogen peroxide removal.<sup>74</sup> Moreover, melanocyte survival after some years has been previously demonstrated.<sup>75</sup> Although measuring NO and TNF levels and viability of skin melanocytes can be useful in confirming the findings, their assessments are considered as part of our limitations. Therefore, further evaluation is needed as a future study, which we hope will be done in the near future. Anyhow, our findings revealed that

aminoguanidine in combination with monobenzone was able to decrease the effect of monobenzone. The findings indicated that iNOS inhibitor could serve as a novel effective therapy against vitiligo by combination of NBUVB therapy. Furthermore, NO agents like L-arginine could markedly increase the effectiveness of phototherapy. Further studies are needed to illustrate expression of an inducible iNOS, NO and TNF levels in mice model for clarification of their role in the pathogenesis of vitiligo. Another approach is necessary to confirm the iNOS implications in the near future: the use of 1400W. These approaches normalize the "non-specific effects" of aminoguanidine. An in-vivo siRNA targeting iNOS could be another way to link the iNOS to vitiligious lesions. Systemic infusion of the drugs could interfere with immune profile of the animal. Supporting data for the immune status would strengthen our results and their interpretations.

### List of acronyms

IFN – interferon

GM-CSF - granulocyte-macrophage colony-stimulating factor

NBUVB - narrow band narrowband ultraviolet B

NO - Nitric oxide

NOS - Nitric Oxide Synthase

eNOS - endothelial NOS

iNOS - inducible NOS

nNOS - neuronal NOS

### Authors contributions

KZ, HM, SKM, and AHP conceived and designed the experiments, analyzed the data and wrote the manuscript and supervised the experiments. All authors read and approved the final manuscript.

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### Conflict of Interest

The authors declare they have no financial, personal, or other conflicts of interest.

### Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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### **iNOS inhibition for vitiligo**

Eur J Transl Myol 29 (3): 251-260, 2019

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