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Cisplatin-Induced Oral Mucositis Prevention: *Nigella sativa*'s Anti-Inflammatory Role through NFκB Pathway

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Abstract

Prevention of Cisplatin-induced oral mucositis (OM) Presents substantial challenges due to its association with oxidative stress, inflammation, apoptosis and NFκB pathway activation. This study aims to evaluate the potential of *Nigella sativa* (NS) extract in inhibiting a cisplatin-induced oral mucositis. Cisplatin-induced oral mucositis was modeled in experimental groups treated with varying doses of NS extract (125, 250 and 500 mg/kg BW), compared to negative controls and N-acetylcysteine (NAC) as a positive control. Expression levels of tumor necrosis factor-alpha (TNF-α) were analysed under Western blot analysis, the p50 and p65 gene expression level was determined by qRT-PCR analysis. NS extract notably inhibited TNF-α expression compared to the negative control group, akin to the positive control (NAC). Additionally, NS extract exhibited a dose-dependent regulation of NFκB pathway subunits (p50 and p65) towards levels closer to the baseline, indicating its potential in modulating the inflammatory pathway. The findings suggest that NS extract possesses promising therapeutic potential in mitigating inflammation and NFκB pathway activation in cisplatin-induced oral mucositis.

Keywords: *Nigella sativa*, Cisplatin, Oral mucositis, NFκB pathway, Inflammation

Introduction

Oral mucositis (OM) is a common and debilitating side effect of systemic oncologic therapy and/or radiation, characterized by acute inflammation of the oral mucosa [1]. It presents clinically as erythematous lesions, erosion and ulceration, and histologically as epithelial layer thickening, inflammatory cell

infiltration and decreased blood vessels [2]. OM significantly impacts patient prognosis, with symptoms such as pain, reduced oral intake, and the onset of local or systemic secondary infections leading to decreased quality of life and treatment adherence issues [3,4]. The incidence of OM is particularly high in patients with head and neck cancer undergoing chemoradiation, especially those with primary tumors in the oral cavity, oropharynx and nasopharynx [5]. Approximately 80 - 100 % of these patients experience OM, with 26.4 % classified as severe [6,7]. Conventional chemotherapy can also result in OM in up to 40 % of cases [8]. OM typically manifests 3 - 4 days post-chemotherapy, with severe cases occurring between days 7 and 14 [9]. Cisplatin, a platinum-based chemotherapy drug, is frequently chosen for its broad utility in both nasopharyngeal and non-nasopharyngeal cases. However, it carries a high risk of inducing mucositis [10]. Cisplatin functions by targeting malignant cells in various cell cycle stages, impeding DNA transcription and replication, but also yields adverse effects such as OM [11]. OM associated with cisplatin is notably more severe in the gastrointestinal tract compared to other platinum-based agents like oxaliplatin and carboplatin [7].

Cisplatin-induced OM is a complex process involving Reactive Oxygen Species (ROS) generation and the activation of transcription factors such as NFκB, crucial in the inflammatory phase [12]. The subunits p50 and p65 of NFκB is a key transcription factor involved in various cellular processes, including inflammation, immune responses and cell growth [13]. The process begins with DNA chain breaks induced by anticancer therapy, followed by ROS formation and the generation of damage-associated molecule patterns (DAMPs), such as Chemotherapy Radiation Associated Molecule Pattern (CRAMP) [14,15]. These initiators trigger biological events, including the activation of key cascades like the NFκB pathway, culminating in the expression of over 200 pro-inflammatory cytokine genes, modulatory cytokines, stress response molecules, and cell adhesion molecules, ultimately leading to apoptosis [12,16]. Despite the high incidence and clinical significance of OM, current management strategies are mostly palliative or supportive care, focusing on pain management, nutritional support and maintenance of good oral hygiene [6,7]. Natural products have shown efficacy in addressing radiation or chemotherapy-induced OM due to their antioxidant and anti-inflammatory functions. *Nigella sativa* (NS) has been used historically for its therapeutic potential and safety [17]. Several studies reported that NS functions as an antioxidant, anti-inflammatory, antibacterial, antifungal and cytoprotective agent [18-20]. Previous study showed that NS's role in managing chemotherapy-induced OM in head and neck cancer patients and animal models [21-23]. However, research has mainly focused on cisplatin-induced gastrointestinal toxicity, leaving a significant gap in understanding mechanisms and developing effective protective strategies or agents, particularly in the oral region.

The significant burden of OM in patients undergoing cisplatin-based chemotherapy, there is a pressing need for effective interventions to prevent or mitigate this debilitating condition. The ethanol extract of NS known for its antioxidant and anti-inflammatory properties, presents a promising candidate for such an intervention. This study aims to evaluate the efficacy of NS ethanol extract in cisplatin-induced OM in animal model.

Materials and methods

Extraction of *Nigella sativa* extract

Nigella sativa seeds were collected from Semarang in Central Java Indonesia in May 2023 (Latitude −7.6565111 and Longitude 109.129500). They were rinsed with tap water followed by distilled water to remove the dirt on the surface. The dried seeds were blended until small pieces and sieved with a mesh size of 120 mesh. The 500 g of NS seeds was extraction in a maceration apparatus with 5 L 98 % ethanol for 24

h. The filtrated was then evaporated under rotary vacuum evaporator (IKA) and the crude extract was kept in refrigerator 4 °C [24,25]. The NS extract was stored at 4 °C until further analysis.

Cisplatin-induced oral mucositis animal model

The study utilized thirty male Wistar rats (250 ± 20 g) maintained under controlled conditions of room temperature (21 °C ± 2), humidity (55 % ± 10) and a 12-hour light-dark cycle, with ad libitum access to food and water. After a week of acclimation, the rats were randomly divided into 6 groups: Healthy, negative control (induced with cisplatin 5 mg/kg BW and received no treatment), N-acetylcysteine (NAC) 500 mg/kg BW and 3 groups receiving different doses of *Nigella sativa* (NS) extract (125, 250 and 500 mg/kg BW) were administered by oral gavage on days 1, 3, 5 and 7. Cisplatin was administered intraperitoneally on days 1, 3 and 5, and the rats were sacrificed on day 8 by dislocation decapitation [18,26].

Protein expression analysis by western blot

The oral mucosa tissue was lysed in RIPA buffer and protein concentration was measured using Pierce BSA CBB Assay. Aliquots of 10 µg total protein were mixed with 2× Laemmeli buffer (Biorad) with ratio 1:1, boiled and separated on 10 % SDS-PAGE gels, transferred to Polyvinylidene Fluoride (PVDF) membranes. Then blocked with 5 % Bovine Serum Albumin (BSA) (Sigma Aldrich, Louis St, MO) in Phosphate Buffered Saline with Tween (PBST) (Sigma Aldrich, Louis St, MO) for 1 h. TNF-α antibodies (Santa Cruz Biotechnology) were applied in blocker overnight at dilutions of 1:1,000 separately, after which membranes were washed, incubated with HRP-conjugated secondary antibody (GeneTex Biotechnology), washed again, incubated with ECL reagent and exposed to chemiluminescence ECL chemiluminescence was captured using the Invitrogen IBright ChemiDoc Imaging System [27-29].

p50 and p65 gene expression by qRT-PCR

Total RNA from rat oral mucosa tissue was extracted with TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesized with 1 g of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse-transcription in a real-time PCR instrument (PCR max Eco 48) and mRNA levels of the p50 and p65 genes were measured using the respective primers (**Table 1**). The thermocycler conditions used were as follows: Initial step at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. The gene expression was recorded as the Cycles threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method) [30-33].

Table 1 Primer sequence.

Gene	Sequence
p50	Forward: TGGACAGCAAATCCGCCCTG Reverse: TGTTGTAATGAGTCGTCATCCT
p65	Forward: TGAACCGAAACTCTGGCAGCTG Reverse: CATCAGCTTGCGAAAAGGAGCC

Statistical analysis

Statistical analyses were accomplished with software SPSS 26.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). Data analysis used 1-way ANOVA and continued with the Least Significant Difference (LSD) test with p-value < 0.05.

Results and discussion

Results

NS extract regulated subunit p50 and p65 NFκB pathway

The mean expression ratio of the p50 gene exhibited a significant decrease in the NAC 500 mg/kg BW group (0.94 ± 0.21), NS 125 mg/kg BW group (0.70 ± 0.24), NS 250 mg/kg BW group (0.67 ± 0.35) and NS 500 mg/kg BW group (1.10 ± 0.34) compared to the negative control group (3.16 ± 0.41) (**Figure 1(A)**). Similarly, the mean expression ratio of the p65 gene also notably decreased in the NAC 500 mg/kg BW group (1.26 ± 0.24), NS 125 mg/kg BW group (0.68 ± 0.39), 250 mg/kg BW group (1.06 ± 0.20) and 500 mg/kg BW group (1.30 ± 0.26) in comparison to the negative control group (2.11 ± 0.38) (**Figure 1(B)**).

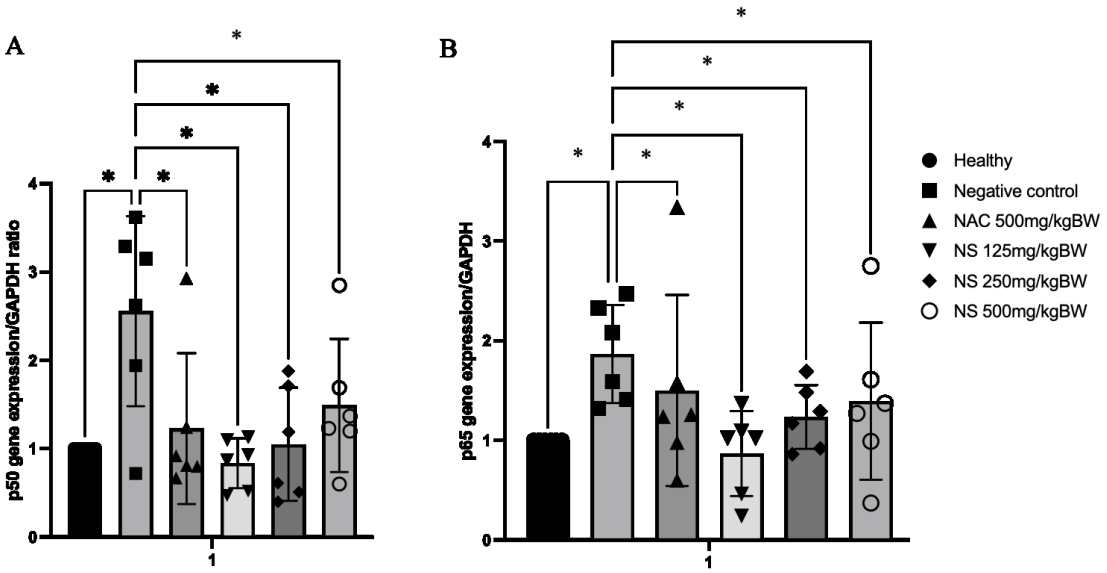

Figure 1 NFκB pathway analysis by qRT-PCR. (A) ratio gene expression level of p50 and (B) ratio gene expression level of p65. Data are presented as mean ± SD with n = 4, *p < 0.03, **p < 0.002, indicates significant difference.

NS extract inhibit TNF-α expression on Cisplatin-induced OM

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that is transcriptionally regulated by the nuclear factor kappa B (NFκB) pathway due to the presence of increased oxidative stress. In this study we also evaluate the effect of NS in the inflammation process. We found that the expression of TNF-α decreased significantly in the NAC 500 mg/kg BW group (34.64 ± 3.12), NS 125 mg/kg BW group (29.23 ± 5.68), NS 250 mg/kg BW group (28.76 ± 4.55) and NS 500 mg/kg BW group (34.33 ± 4.44) compared to the negative control group (52.93 ± 3.47) (**Figure 2**).

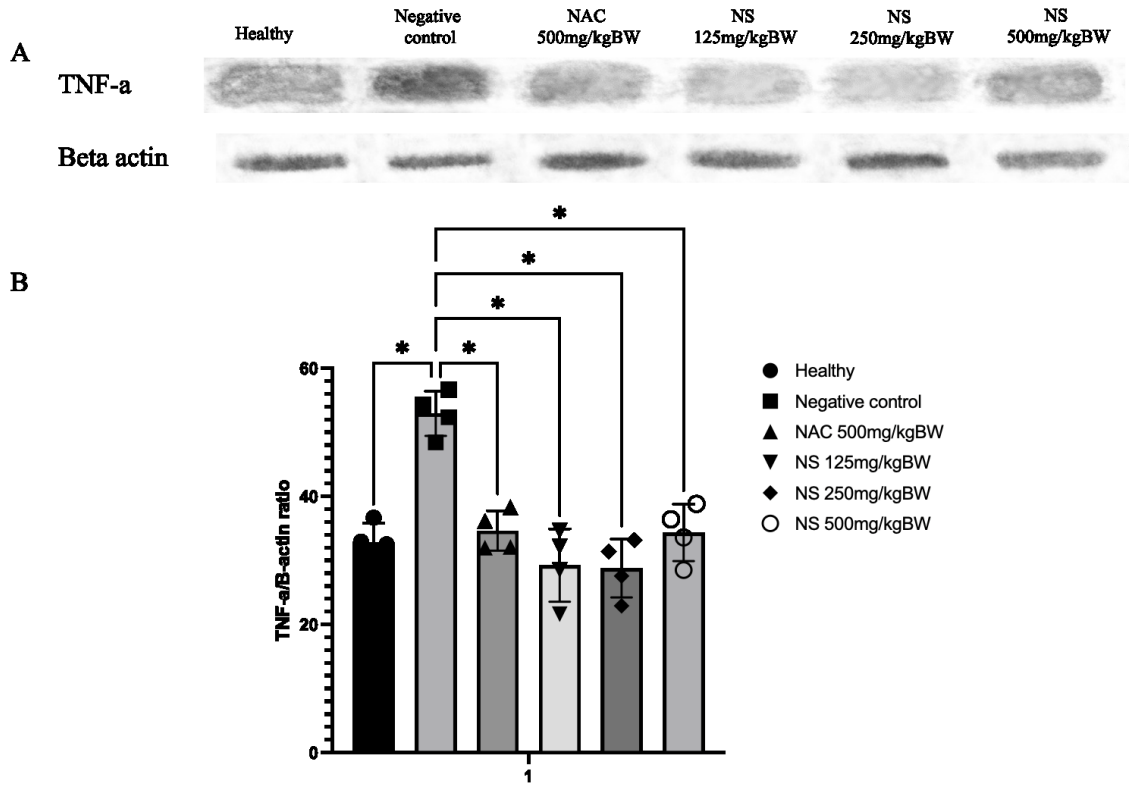

Figure 2 Expression of TNF-α among different study groups in Cisplatin-induced MO. (A) Band intensity of TNF-α and B-actin in Western blot analysis. (B) Quantification of the TNF-α expression ratio to B-actin. Data are presented as mean ± SD with n = 4, ***$p < 0.0002$, ****$p < 0.0001$ indicates significant difference.

Discussion

Oral mucositis (OM) is the inflammation of the oral mucosa commonly found in patients undergoing anticancer chemotherapy [2,7]. OM is associated with increased levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and oxidative stress markers [4,16,34]. The use of natural compounds containing various secondary metabolites such as flavonoids, alkaloids, saponins and tannins from *Nigella sativa* (NS) has been reported to have both anti-inflammatory and antioxidant effects [17-23,35]. However, its role in OM has not been fully elucidated. The NS metabolite compounds can affect the regulation of the p50 and p65 subunits. This study also yielded results that support previous research, demonstrating that NS doses ranging from 125 to 500 mg/kg BB can suppress the expression of the p50 and p65 genes. This proves that NS compounds can inhibit NFκB activation. The suppression of NF-kB activation by thymoquinone has been correlated with the sequential inhibition of IκBα kinase activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, nuclear translocation of p65 and the expression of NFκB-regulated reporter genes [36]. Thymoquinone has been found to specifically inhibit the direct binding of nuclear p65 and recombinant p65 to DNA, and this binding can be reversed by reducing agents, indicating that thymoquinone can modify cysteine residues in p65 [37]. This indicates that thymoquinone exerts its regulatory effect on the NFκB pathway by inhibiting key signaling molecules and suppressing NFκB-regulated gene products [38].

Previous studies have reported that clinical trials using NS oil mouthwash improved chemotherapy-induced oral mucositis in acute myeloid leukemia patients, leading to a subsequent reduction in pro-inflammatory cytokine levels such as TNF-α [19,37]. The findings of these studies are consistent with the

results of the current study, where NS administration suppressed TNF-α expression. Notably, the 125 mg/kg BW dose of NS exhibited the most significant suppression of TNF-α expression. The potential mechanism behind this effect could be attributed to the anti-inflammatory and antioxidant properties of NS. The phytochemical content of NS, including thymoquinone, has been reported to possess anti-inflammatory and antioxidant properties [39]. Thymoquinone in NS has also been shown to inhibit TNF-α-induced inflammation through various mechanisms, such as regulating the apoptosis signal-regulating kinase 1 (ASK1) pathway and inhibiting the activation of p38 and JNK, which are involved in the inflammatory signaling cascade [36]. These findings indicate that thymoquinone provides anti-inflammatory effects by modulating intracellular signaling pathways associated with inflammation and suppressing TNF-α. In this study, the 125 mg/kg BW dose of NS extract was more potent in suppressing p50/p65 and TNF-α compared to the 250 and 500 mg/kg BW doses in a cisplatin-induced oral mucositis model. The effects of different doses of NS extract can be influenced by various factors, including the pharmacokinetics of the compounds, bioavailability and their specific interactions with the molecular pathways involved in the regulation of p50/p65 and TNF-α [40].

Conclusions

Overall, the administration of NS extract therapy shows potential in reducing inflammation in oral mucositis by suppressing pro-inflammatory cytokines such as TNF-α and preventing cell death through NFκB pathways. Further research is needed to explain the specific mechanisms underlying the effects of *Nigella sativa* on oral mucositis due to cisplatin.

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