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Article

Effect of quercetin and role of nitric oxide pathway in chloroquine-induced scratching

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Nitric oxide (NO) is an abundant mediator which is demonstrated to be involved in pruritus. Assuming that the increased NO also mediates chloroquine-induced pruritus, which is a frequent complication seen in the chronic chloroquine treatment, the current study aimed to investigate the effect of quercetin and the role of NO in chloroquine-induced pruritus in C57BL/6 mice. Model was created with subcutaneous chloroquine (400µg/site) injection to the nape of the mice. Effect of quercetin and role of NO were investigated with administration of quercetin, and co-administration with L-NAME, 7-NI and L-arginine before chloroquine injection. Locomotor activity was assessed by activity cage and number of the scratching bouts after chloroquine injection was recorded for 30 minutes. Our results show that quercetin significantly reduced scratching bouts at the doses of 10, 20, 40 and 80 mg/kg. Locomotor activity was decreased at the 40 and 80 mg/kg doses of quercetin. Additionally, decrease of the number of scratching bouts by quercetin prevented by L-arginine treatment, while L-NAME and 7-NI enhanced the anti-pruritic effect of sub-effective doses of quercetin. Therefore, our study demonstrated that acute injection of quercetin significantly diminished chloroquine-induced scratching behavior, and this effect is partly mediated by inhibition of neuronal nitric oxide synthase enzyme.

Keywords: Chloroquine-induced itch. Quercetin. Nitrergic system. Scratching.

INTRODUCTION

Pruritus is generally defined as an unpleasant itching sensation that leads to scratching(Furue *et al.* 2020). The condition is one of the most frequent complaints in dermatology clinics and can be the result of different metabolic or internal organ-related diseases(Miyahara *et al.* 2020). This debilitating symptom has an obvious effect on the quality of life of patients. Although anti-histaminergic drugs are the first option against acute and chronic pruritus treatment, opioid system antagonists are also used in particular cases(Ko 2014). But there is still a need for basic and translational research for the identification of better treatment options for the histaminergic and non-histaminergic pruritus.

Different hypotheses other than the histaminergic pathway have been extensively investigated and various targets have been identified in the last decades (Reszke, Krajewski,Szepietowski 2020). In this context, the chloroquine-induced pruritus for identification of novel treatment options for the histamine-independent itch is frequently used. Besides its main use in the treatment of certain types of malaria, chloroquine has long been used for amebiasis, rheumatoid arthritis, systemic lupus erythematous and as a radio-sensitizing agent against several types of malignancies(Dos Reis Neto *et al.* 2020). However, acute intradermal or subcutaneous injections of chloroquine induce a strong pruritic response via the Mas-related G-protein coupled receptor member-A3(MrgprA3) activation(Tarrason *et al.* 2017). Chloroquine activates $G_{\beta\gamma}$ coupling and modulates transient receptor potential cation channel (TRPA1) dependent itch(Wilson *et al.* 2011). Additionally, this coupling modulates ion channels and neuropeptides such as N-methyl-D-aspartate (NMDA) and substance P, which have a role in the pruritus mechanism.

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Furthermore, activation of this mechanism also results in increased nitric oxide (NO) production, which elicits pruritus mediated by increased NO in dermal tissue and central nervous system(Tarrason *et al.* 2017). Considered together, chloroquine-induced NO dependent pruritus is suitable for the investigation of novel compounds in the non-histaminergic itch pathology.

Quercetin is one of the flavonoid compound which have the high antioxidant capacity with anti-inflammatory and anti-allergic actions(Anand David, Arulmoli,Parasuraman 2016). These actions are generally related to high antioxidant and anti-inflammatory effects, free radical scavenging properties, interference of nitric oxide synthase, inhibition of xanthine oxidase and tumor necrosis factor-α (TNF-α) release (Li *et al.* 2016). The anti-pruritic effect of quercetin is investigated in cosmetic products for the human body(Maramaldi *et al.* 2016, Jafarinia *et al.* 2020). Therefore, it is rational to think that its effect on the nitric oxide system and anti-allergic actions, quercetin could be protective against NO-mediated pruritus in the chloroquine-induced experimental scratching model.

Knowledge regarding the effects of quercetin in experimental pruritus remains limited, therefore in this study, we sought to investigate its effects on chloroquine-induced pruritus. Since nitric oxide is the main mechanism of chloroquine-induced pruritus and quercetin possibly influences this mechanism, our results provide mechanistic insights into the action of quercetin in chloroquine-induced pruritus and will provide information for further studies.

MATERIAL AND METHODS

Animals

Experiments were conducted with male C57BL/6 mice (n=100). Animals were obtained from Ondokuz Mayıs University vivarium after approval from the University's Experimental Animals Ethics Committee (HADYEK 2020/44). Mice were maintained in groups of 5 mice per cage under standard conditions (22±2°C, 55% humidity, 12-12 day and night cycle) and fed *ad libitum*. All treatments were performed according to the Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize animal suffering.

Chemicals

Quercetin dihydrate, chloroquine diphosphate, L-arginine, and L-NAME were purchased from Sigma Aldrich In. (Illinois, US). 7-nitroindazole (7-NI) were obtained from Tocris Bioscience (Bristol, UK). Quercetin dihydrate and 7-nitroindazole were dissolved in phosphate buffered saline (PBS): dimethyl sulfoxide (DMSO) (5:1, v/v). L-arginine, N(ω)-nitro-L-arginine methyl ester (L-NAME), and chloroquine diphosphate were dissolved in the saline (0.9% NaCl) solution. All drugs were freshly dissolved before the experiments and administered intraperitoneally, except chloroquine and 7-NI.

Locomotor activity

Animals were habituated to the laboratory environment for three days before the experiments. In order to investigate the effects of the drug treatments on motor coordination, mice were tested with a locomotor activity cage, which consisted in vertical and horizontal grids, which count animal movements for the selected period. Animals were placed on the locomotor apparatus after drug treatments, in accordance with the pharmacokinetic knowledge present in the literature(Bush,Pollack 2001, Heinzen,Pollack 2003, Goromaru *et al.* 2005, Yin *et al.* 2019). Afterwards, animals were tested for 5 minutes to investigate the effects of drug treatments on locomotor behavior.

Experimental design

Prior to the chloroquine injection, the rostral part of the animals was shaved with depilatory cream (Veet®, Reckitt Benckiser, UK). Pruritus was induced by subcutaneous administration of chloroquine (400 µg/mouse) to the nape of the animals, as previously described. Quercetin (5, 10, 20, 40, 80 mg/kg, i.p.) was administered thirty minutes before the chloroquine injection. To investigate the possible role of NO on the effect of quercetin, L-arginine (10,30 and 100 mg/kg, i.p.),

L-NAME (1,5 and 10 mg/kg, i.p.) and 7-NI (0.16, 0.8 and 1.6 µg/kg, intradermal) were co-administered before the chloroquine injection. Effective and sub-effective doses of L-NAME, L-arginine and 7-NI were selected based on the previous reports for combination experiments(Haddadi *et al.* 2020). L-NAME, L-arginine and 7-NI were co-administered with quercetin, thirty minutes before the chloroquine injection. After the chloroquine injections, animals were placed in an open plexiglass apparatus and recorded with a camera from two angles for 30 minutes, by two blinded observers. Each bout was accepted as the lifting of the hind paw to the area of injection and returning to the floor or the mouth of the animal. At the end of the experiments, the videos were played back and the number of the scratching bouts at the site of the injection was quantified by two blinded observers.

Statistical analysis

All experimental data were analyzed with SPSS (v21.0, IBM, US). All data were expressed as mean ± standard error of the mean (SEM). After the determination of data distribution with normality test, one-way ANOVA for normal distribution or Kruskal-Wallis for non-normal distribution were performed. Multiple comparisons were performed with the Mann-Whitney U, Tukey, or Tamhane's T2 tests. P values less than 0.05 were considered significant.

RESULTS

Effect of quercetin on locomotor activity

Locomotor activity was evaluated with an activity cage. Quercetin 5 (346±8.44), 10 (338±9.33) and 20 mg/kg (341±8.85) did not significantly affect locomotor activity compared to control (365±4.74) (ANOVA, $F(5,36)=112$, $p>0.05$, Figure 1). But quercetin at the doses of 40 (126±18.9) and 80 (78.1±16.2) mg/kg significantly decreased locomotor activity compared to control (Kruskal-Wallis, $\chi^2=30,8$, $p<0.001$, Figure 1).

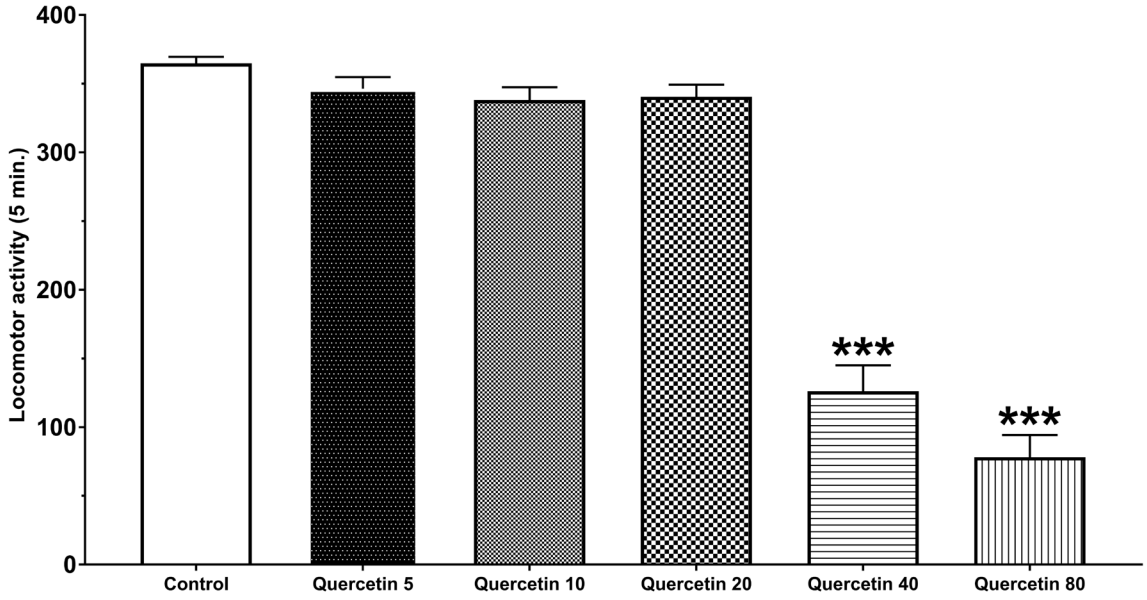

FIGURE 1 - Locomotor activity results of all experimental groups. Quercetin decreased locomotor activity at 40 and 80 mg/kg doses. All data represented as mean±SEM. ***p<0.001 versus the control group.

Quercetin decreased the number of the chloroquine-induced scratching bouts

Scratching bouts were counted by two blind observers. Chloroquine (65.4±7.96) significantly caused scratching bouts compared to the control (4.68±0.67) (ANOVA, F(6,42)=39.8, p<0.001, Figure 2). Quercetin at the dose of 5 mg/kg (66.4±6.57) did not significantly affect chloroquine-induced bouts compared to the sole chloroquine treatment (ANOVA, F(6,42)=39.8, p>0.05). But quercetin 10 (21.1±4.86), 20 (24±1.66), 40 (4.57±0.649), and 80 (4.29±0.606) significantly attenuated the number of scratching bouts compared to the chloroquine (ANOVA, F(6,42)=39.8, p<0.001, Figure 2). Additionally, the preventive effect of quercetin was found to be stronger at the doses of 40 and 80 mg/kg compared to the quercetin 10 and 20 mg/kg treatments (ANOVA, F(6,42)=39.8, p=0.45, p=0.40, respectively) (Figure 2).

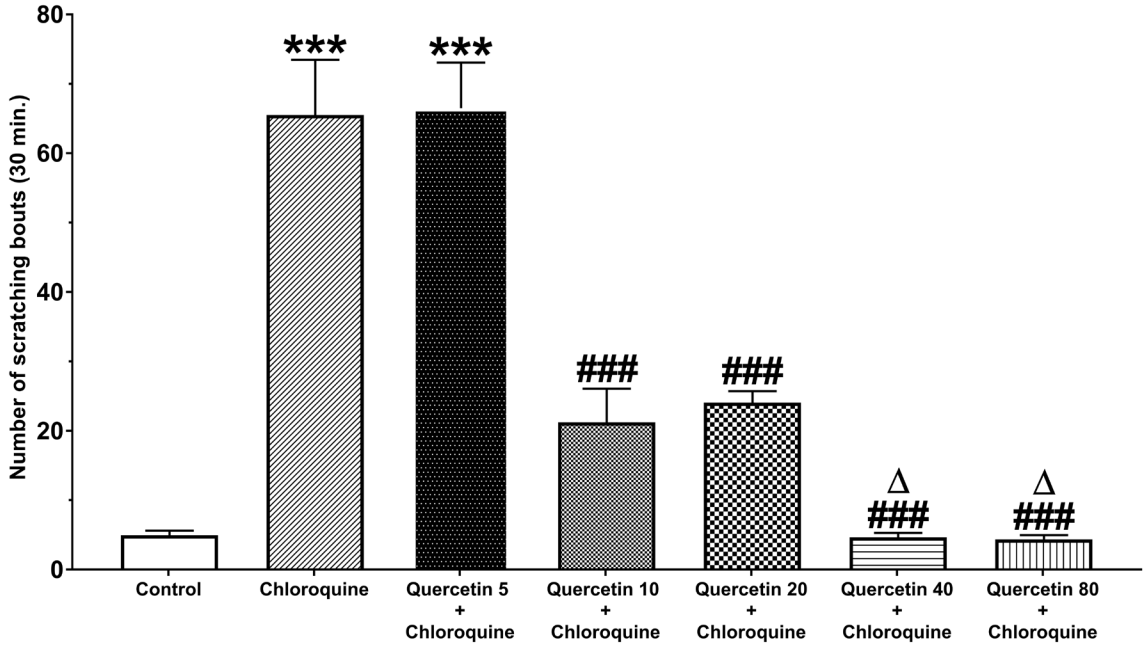

FIGURE 2 - Effect of quercetin on chloroquine-induced increase in the number of scratching bouts. All investigated doses significantly suppressed chloroquine-induced scratching except 5 mg/kg treatment. But that suppressive effect was found to be stronger in the 40 and 80 mg/kg doses. All data represented as mean±SEM. ***p<0.001 versus control and ###p<0.001 versus chloroquine and Δp<0.05 versus quercetin 20 mg/kg group.

Nitric oxide pathway has a role in chloroquine-induced scratching

Before the evaluation of the role of the nitrergic system in the anti-pruritic effect of quercetin, effects of different doses of L-NAME (1, 5, and 10 mg/kg), 7-NI (0.16, 0.8, and 1.6 µg/kg), and L-arginine (10, 30 and 100 mg/kg) were investigated. The non-selective neuronal nitric oxide synthase (nNOS(inhibitor L-NAME at the dose of 1 mg/kg (88.14±2.13) did not significantly affect chloroquine-induced increases in the scratching bouts. However, at the doses of and 5 mg/kg (49.85±4.04) and 10 mg/kg, L-NAME (16.14±3.34) significantly prevented chloroquine-induced increases in the number of the scratching bouts compared to the chloroquine (ANOVA, F(4,30)=92.1, p=0.001, p<0.001, respectively)(Figure 3). Next, we examined the effect of nNOS chloroquine-induced bouts evaluated with 7-NI administration, and

our results showed that 7-NI at the doses of 1.6 µg/kg (19.14±2.19) and 0.8 µg/kg (37.71±3.80) significantly ($p<0.001$) decreased the number of the scratching bouts, except at the dose of 0.16 µg/kg (78.85±4.29) which failed to show a significant difference compared to chloroquine treatment (ANOVA, $F(4,30)=71.9$, $p>0.05$) (Figure 3). In contrast to the NOS inhibitors L-arginine, a well-known precursor of nitric oxide (NO), significantly potentiated chloroquine-induced scratching responses at the dose 100 (122.57±7.27) and 30 mg/kg (102±5.48) ($p<0.001$, $p=0.03$, respectively) (Figure 3). But this effect was absent at the 10 mg/kg (122.57±7.27) dose of L-arginine compared to the chloroquine (ANOVA, $F(4,30)=58.9$, $p>0.05$) (Figure 3).

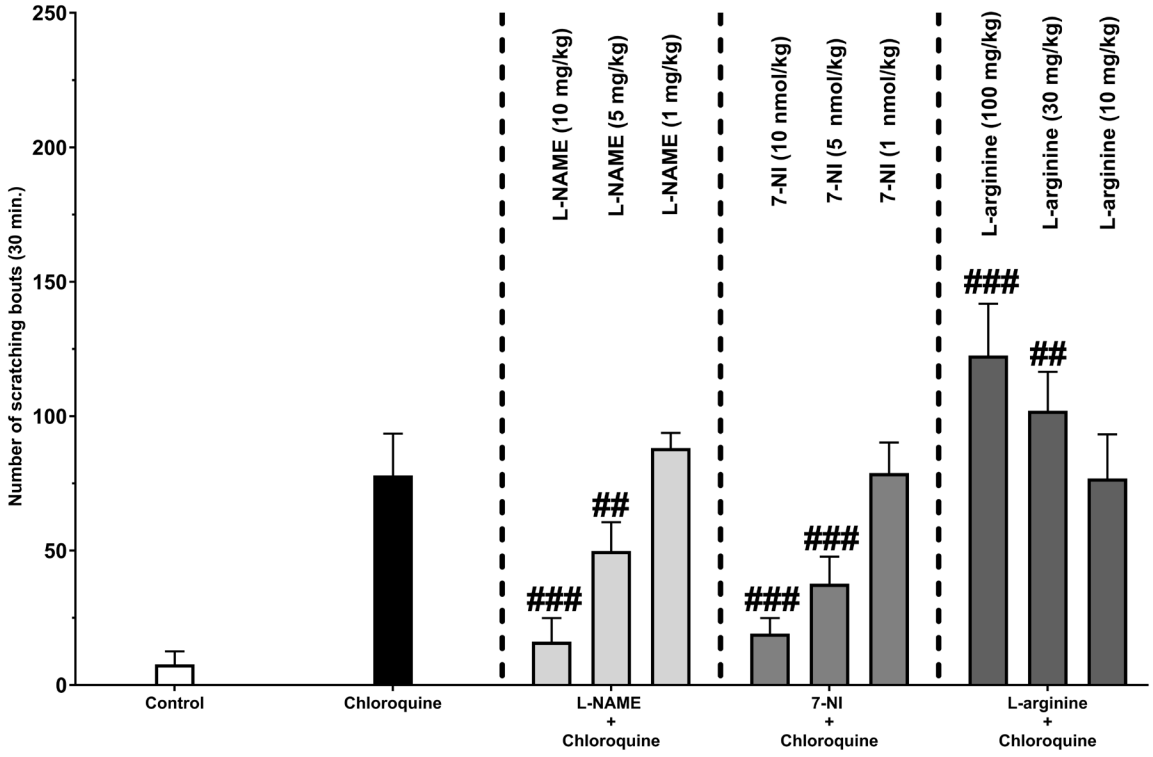

FIGURE 3 - Investigation of the possible role of NO in chloroquine-induced pruritus. Inhibition of NOS (L-NAME at 10 and 5 mg/kg) and nNOS (7-NI at 1.6 and 0.8 µg/kg) significantly decreased the numbers of chloroquine-induced scratching bouts. L-arginine (100 and 30 mg/kg) significantly enhanced the number of chloroquine-induced scratching bouts. All data represented as mean±SEM. ###$p<0.001$, ##$p<0.01$ versus chloroquine group.

Inhibition of NOS has a role in the preventive effect of quercetin against chloroquine-induced pruritus

Evaluation of the anti-pruritic effect of quercetin was employed with co-treatment of quercetin with effective and sub-effective doses of L-NAME, 7-NI, and L-arginine (Figure 4). Firstly, a sub-effective dose of quercetin (5 mg/kg) was co-administered with a sub-effective dose of non-selective NOS inhibitor, L-NAME (10 mg/kg) before the chloroquine treatment. This combination significantly decreased the number of scratching bouts compared to the chloroquine treatment (39±1.77) (ANOVA, $F(6,42)=78,5$, $p<0.001$, Figure 4). Secondly, a sub-effective dose of quercetin was co-administered with a sub-effective dose of selective nNOS inhibitor, 7-NI, before the chloroquine treatment (Figure 4). Our results demonstrated that co-

administration of sub-effective doses of quercetin with 7-NI (19.4±1.67) significantly attenuated chloroquine-induced scratching response (ANOVA, F(6,42)=78,5, p<0.001, Figure 4), even more strongly than compared to the quercetin 5 mg/kg and L-NAME 1 mg/kg combination (ANOVA, F(6,42)=78,5, p<0.001, Figure 4). Thirdly, the effect of quercetin was investigated with NO potentiator L-arginine (Figure 4). Co-administration of effective dose of L-arginine (10 mg/kg) with effective dose of quercetin (10 mg/kg) reversed anti-pruritic effect of quercetin (ANOVA, F(4,30)=58.9, 76.6±3.34, p>0.05) (Figure 4). Additionally, co-administration of an effective dose of L-arginine with a sub-effective dose of quercetin (5 mg/kg) potentiated chloroquine-induced number of scratching bouts (124±3.88), which was significant compared to the chloroquine group (ANOVA, F(4,30)=58.9, p<0.001, Figure 4).

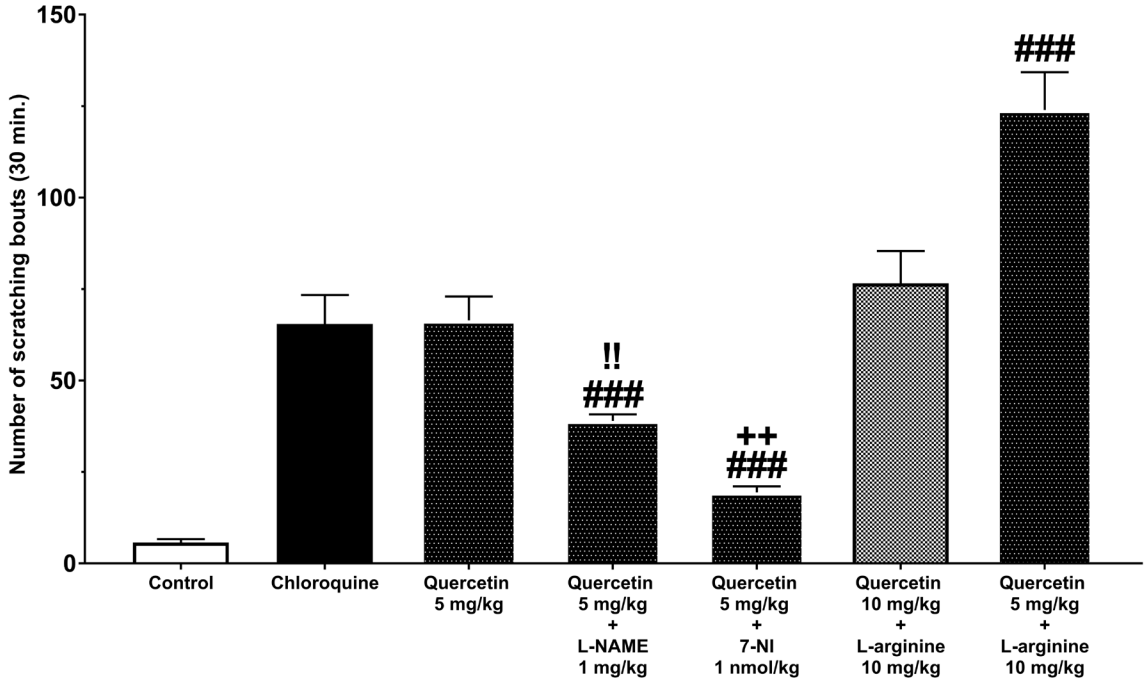

FIGURE 4 - Evaluation of the possible role of NO in suppressive effect of quercetin. But co-administration of the sub-effective dose of quercetin with sub-effective doses of L-NAME (1 mg/kg) and 7-NI (0,16 µg /kg) significantly suppressed the chloroquine-induced number of the scratching bouts. Additionally, a sub-effective dose of 7-NI more significantly decreased the number of chloroquine-induced scratching bouts, while an effective dose of L-arginine (10 mg/kg) significantly enhanced the number of chloroquine-induced scratching bouts when co-administered with a sub-effective dose of quercetin (5 mg/kg). All data represented as mean±SEM. All data represented as mean±SEM. ###p<0.001 versus chloroquine and !!p<0.01 versus quercetin (5 mg/kg) and !!p<0.01 versus quercetin (5 mg/kg) + 7-NI (0,16 µg/kg) group.

DISCUSSION

Our results demonstrated that quercetin doses dependently suppressed chloroquine-induced scratching. Increasing NO with L-arginine, which is precursor of NO synthesis, were shown to enhance chloroquine-induced pruritus, as previously demonstrated (Haddadi *et al.* 2020). But dose-dependent inhibition of inducible nitric oxide synthase (iNOS) and nNOS was showed to be inhibited chloroquine-induced pruritus. Additionally, a combination of sub-effective doses of quercetin with these inhibitors showed that the nitric oxide pathway participates in the anti-pruritic effect of quercetin, in chloroquine-induced scratching.

According to the oral toxicity tests after the quercetin treatment, a 80 mg/kg dose of quercetin was selected as

the highest dose in our experiments(Chen *et al.* 2014). Before the investigation of the possible effects of quercetin and the role of the nitrergic system, we performed our experiments with 5, 10, 20, 40, and 80 mg/kg doses of quercetin to demonstrate the effects of different treatment doses. Although 10, 20, 40 and 80 mg/kg significantly suppressed chloroquine-induced scratching, 40 and 80 mg/kg doses decreased locomotor activity. Because it is important to evaluate locomotor activity of the treated drugs to discriminate if anti-pruritic effect in relation with decreased locomotor activity, 40 and 80 mg/kg doses were excluded from further experiments. According to the previous studies, it has been reported that quercetin decreases locomotor activity the doses of 30 mg/kg when administered intraperitoneally or subcutaneously (Kanazawa *et al.* 2016, Kanazawa *et al.* 2017). Therefore, we investigated the role of the nitrergic system with 5 and 10 mg/kg doses of quercetin, which were the sub-effective and least effective doses.

NO is a gaseous signaling molecule that plays various roles in human physiology(Panthi,Gautam 2017). Besides its neuromodulator and neurotransmitter actions, its part in pruritus has been extensively investigated, and several reports underline that the effect of NO in pruritus occurred by chloroquine, serotonin and substance P(Andoh,Kuraishi 2003, Foroutan *et al.* 2015, Ostadhadi *et al.* 2017). Notably, it was shown that pruritus resulting from substance-P and serotonin was exacerbated by NO treatment in mice(Akiyama,Carstens 2013). Scratching behavior in humans and rodents was successfully imitated by chloroquine via a histamine-independent pathway. Injection of chloroquine to the nape of the mice is frequently used for mimicking the clinical aspect of chloroquine-MrgprA3-NO-dependent pruritus(Hassanipour *et al.* 2016). Foroutan *et al.* (2015) as well as Ostadhadi *et al.* (2017), also demonstrated that NO/cyclic guanosine monophosphate participates in chloroquine-induced pruritus in an L-NAME and selective nNOS inhibitor suppressible manner. Ostadhadi *et al.* (2017),supported the view that NOS is present in the itch sensation pathway cells which are C-fibers, spinal cord and brain(Davidson,Giesler 2010). Additionally, increased serum and local concentration of NO in the pruritus in some skin disorders, such as psoriasis and atopic dermatitis, were demonstrated by several groups(Andoh,Kuraishi 2003). Inhibition or prevention of this increase was NO synthase inhibitor L-NAME attenuated pruritus in atopic dermatitis and psoriasis(Domagala, Szepietowski,Reich 2017, Lee *et al.* 2020). Considered together, NO and pathways that play a role in the synthesis or degradation of NO also play an important role in chloroquine-induced pruritus. But Foroutan *et al.* (2015),also suggested that while intradermal injection of L-NAME and 7-NI decreases scratching response, aminoguanidine as iNOS inhibitor could not reduce it. According to the nitric oxide synthesis mechanism described, intracellular calcium is essential for stimulation of nNOS and eNOS, but not iNOS. In relation to this, increased intracellular calcium levels in neurons after chloroquine were demonstrated(Forstermann,Sessa 2011). Therefore, it is possible that nNOS and eNOS but not iNOS, could be involved in chloroquine-induced NO production, which also explains the absence of the effect of aminoguanidine. Based on that knowledge, we used 7-NI to investigate possible role of nNOS. It was previously demonstrated that increased epidermal level of NO by nNOS after substance p injection is crucial for scratching(Andoh,Kuraishi 2003). Although, 7-NI also inhibits eNOS at higher concentrations, it is important to consider the possible role of eNOS inhibition on chloroquine-induced pruritic behavior. Further studies demonstrated that the effect of 7-NI on eNOS is a hundred-fold dose-dependent than on nNOS, and it does not seem rational to reach these levels with an intradermal injection(Yu *et al.* 2011). Therefore, the effect of 7-NI is relatively selective on nNOS in the neurons of the peripheral nervous system when administered intradermally. Our results, in line with these studies, showed a L-NAME and 7-NI inhibited chloroquine-induced increase in the number of scratching bouts, dose-dependently.

Quercetin is a natural dietary flavonoid present in fruits and vegetables. Recent studies demonstrated the antioxidant, anti-inflammatory and neuroprotective action of quercetin *in vivo* and *in vitro*(Xu *et al.* 2019). *In vitro* studies demonstrated the suppressive effect of quercetin treatment on nasal epithelial and lipopolysaccharide(LPS)-stimulated macrophages(Ebihara *et al.* 2018). Recently, Jafarinia *et al.* (2020),demonstrated that quercetin inhibited substance P, calcitonin gene-related peptide and nerve growth factor, in addition to the decreased NO levels in the allergic rhinitis model in rats. Jafarina broadly

investigated anti-allergic effects of quercetin. But anti-allergic and pruritus related effects of quercetin were mostly investigated in vitro. Therefore, we chose to investigate possible anti-pruritic effects of quercetin in living animals, which is seen in inflammatory/allergic diseases such as psoriasis. Our results demonstrated that quercetin showed an anti-pruritic effect, supporting the results of the soothing and anti-pruritic effect of quercetin demonstrated in a single-blind study(Maramaldi *et al.* 2016).

However, based on the role of NO in chloroquine-induced pruritus, we also investigated the role of NO on the anti-pruritic effect of quercetin. Several flavonoids have already been demonstrated as nitric oxide modulators. A wide range of reports demonstrated a possible relationship of quercetin with the nitrergic system(Kao *et al.* 2010). Additionally, the effect of quercetin on nitric oxide synthase and reactive nitrogen species showed itself in living animals(Cho,Kim 2013). However, knowledge about the exact interplay with the nitrergic system is still limited. Taguchi *et al.* (2020),demonstrated that quercetin rescued nitric oxide production by promoting endothelial nitric oxide synthase (eNOS) in endothelial cells, while Lee *et al.* (2020),demonstrated quercetin suppressed LPS-induced iNOS increase and neuroinflammation in Sprague-Dawley rats. Supporting NO suppressor effect of quercetin, Güran *et al.* (2019), demonstrated quercetin decreased NO production. Elsewhere, Ebihara *et al.* (2018),demonstrated quercetin suppresses nitric oxide production in nasal epithelial cells. Also, Kao *et al. (2010)*, demonstrated that quercetin treatment inhibited NO production in endotoxin/cytokine-stimulated microglia and downregulated cellular iNOS expression. Our results demonstrated parallel results with these studies in which quercetin prevented NO-mediated effects. Additionally, we showed that anti-pruritic effect of quercetin was reversible with L-NAME and 7-NI treatments. This suppressive effect was stronger in the 7-NI treatment compared to L-NAME co-administration with quercetin. Thus, suppression of the NOS pathway, presumably trough nNOS, has a role in the anti-pruritic effect of quercetin. Another important enzyme that should not be forgotten, possible role of eNOS. Our results could be explained two ways considering possible role of eNOS. First, though there are several studies which suggest quercetin inhibits eNOS, they only investigated this inhibitory effect on isolated tissues, which might be differ from systemic treatment seen in our study. Secondly, we only investigated a combination of quercetin with the eNOS inhibitor in the sub-effective and least effective doses, due to the decreased locomotor activity in the higher doses. Thus, we thought that the possible inhibitory effect of quercetin on eNOS is dose dependent as well as related to decreased locomotor activity, as seen in our study. No doubt these two explanations require further investigations to confirm our results.

In conclusion, our results demonstrated that quercetin has an anti-pruritic effect in the chloroquine-induced scratching model and that this effect of seems to involve the nNOS pathway. However, though our study demonstrated the nNOS-mediated anti-pruritic effect of quercetin, further studies should be performed to investigate the effect of quercetin on serum and skin levels of NO in chloroquine-induced itch.

DECLARATIONS

This work is not supported by any foundation.

CONFLICT OF INTEREST

The authors declare there are no competing interests.

ETHICS APPROVAL

Experiments were conducted on one hundred male C57BL/6 mice. Animals were obtained from Ondokuz Mayıs University vivarium after approval from the University's Experimental Animals Ethics Committee (HADYEK 2020/44). Mice were maintained in groups of 5 mice per cage under standard conditions (22±2°C, 55% humidity, 12-12 day and night cycle) and fed *ad libitum*. All treatments were performed according to the Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize animal suffering.

Authors' contributions

Conception and design: OK and CG. Data acquisition, data analysis and interpretation: CG.

Drafting the article or critically revising it for important intellectual content: OK. Final approval of the version to be published: OK and CG.

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