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Original Article

Quinine Attenuates Cigarette Smoke Extract Induced Mucosal Inflammation and Oxidative Stress in the Zebrafish Model

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ABSTRACT

Introduction: Smoking is the primary cause of Chronic obstructive pulmonary disease (COPD) leading to alarming increase in worldwide death. Cigarette smoking is a dangerous risk factor in causing mucosal inflammation and lung damage caused by nicotine, which is more addictive than other narcotics. This study was undertaken to assess the potential of quinine in ameliorating the cigarette smoke extract (CSE) induced inflammation in the Zebrafish model. **Materials and Methods:** CSE was extracted in water and the Zebrafish was exposed to concentrations ranging from 0.2 to 1.0 cig/L. IC_{50} of CSE was found to be 0.3 cig/L and this concentration was used for further studies. Induced Zebrafish were treated with 5 mg, 10mg and 15 mg/kg of quinine and analysed for reactive oxygen species, nitric oxide levels, myeloperoxidase activity, histopathology and expression of pro-inflammatory (IL-1) and anti-inflammatory (IL-10) cytokine. **Results:** The results indicate that exposure of Zebrafish to CSE has significantly increased the levels of ROS, MPO, NO and pro-inflammatory cytokine. Upon treatment with quinine, a dose dependent decrease in these levels was observed with the significantly higher decrease in 15mg/kg treated animals for 8 days. The levels of the anti-inflammatory cytokine, IL-10 tend to be increased with increasing concentration of quinine. The histopathological examination has shown inflammation and damaged gill filaments in the CSE exposed group the Gills inflammation and damaged gill filaments with increased neutrophil accumulation whereas these changes are not observed in the quinine-treated animals. **Conclusion:** Cumulatively, the results obtained in this study confirm the role of quinine in ameliorating the CSE-induced inflammation in the Zebrafish model.

Keywords: Cigarette smoke extracts, Mucosal inflammation, Quinine, ROS, Zebrafish.

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INTRODUCTION

Tobacco smoking serves as a major causative factor number of ailments including respiratory and heart disease and cancer.[1] Cigarette smoke has thousands of toxic chemicals among which many chemicals have been reported to induce inflammation leading to complex diseases including endocrine effects, neurological disorders and cerebral aneurism.[2,3] Most of the studies on the toxicity of cigarette smoke, the effect of anti-oxidants, and anti-inflammatory drugs against cigarette smoke have been carried out in a variety of animal models including mice and rats.

Zebrafish is gaining much attention in accessing the toxicity of drugs, screening of candidate molecules, the pathophysiology of diseases their mechanisms genetic changes associated with the pathophysiology of the disease, and screening of antioxidant and anti-inflammatory diseases.[4-6] This is particularly possible due to their smaller size and transparent in nature. The smaller size makes the experiment feasible within a small lab space and small volume of test articles.[7]

Quinine is the most popular anti-malarial drug and serves as the most widely used drug against malarial since its time of introduction.[8] However, the effect of quinine against cigarette smoke-induced inflammation has not been previously investigated. Hence, the present study aimed to investigate the ameliorative effect of quinine against cigarette smoke-induced inflammation in the Zebrafish model.

MATERIALS AND METHODS

Collection and Maintenance of Zebrafish

All Zebrafish used for the experiment were acclimatized for 60 days to the laboratory conditions by maintaining them in a 55 L glass tank filled with dechlorinated tap water. Continual aeration

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was ensured by the use of an air compressor. Temperature and pH of the water as maintained between 26 and 27 degrees Celsius and 7.3 and 7.5, respectively during acclimatization and throughout the experimental period. Animals in all the groups were with commercial food pellets at the frequency of twice a day.

Preparation of cigarette smoke extract (CSE)

Cigarette smoke extraction setup and extraction protocol were based on the previously reported method by, Ellis LD, *et al.* and Gellner CA, *et al.*[1,9] without any modifications. Briefly, the cigarette is connected to one end of the transparent tube which is immersed in 35 ml of 50 ml tightly closed centrifuge tube. Puff was simulated through the use of a syringe connected to another un-immersed transparent tube. All the smoke arising from the cigarette was extracted in the water at the centrifuge tube. A detailed setup was provided in Figure 1.

The inflammatory mediators IL-1β increased and IL-10 decreased significantly in the CSE-treated group. Inflammatory marker, IL-1β decreased and IL-10 increased in a dose-dependent manner following quinine administration of CSE-induced zebrafish.

Determination of LC_{50} value for Cigarette (Cig) smoke extract and Gill inflammation induction

Collected CSE was filtered using Whatman No 1paper and stored at 4°C until further use. LC_{50} determination was carried out by exposing the Zebrafish for 7 days at varying concentrations ranging from 0.2, 0.4, 0.6, 0.8, 1.0 cigarette/L (Figure 2). Fifty Zebrafishes were used for this LC_{50} determination where ten fishes were exposed to each concentration.

Determination of LC_{50} Value Quinine

In order to determine the LC_{50} concentration of quinine, 10 Zebrafishes were exposed to 50 mg/kg body weight for 7 days. In the initial phase, the fishes were fed with 2 to 10 mg/kg of quinine via oral gavage. No toxic response was observed up to 10 mg/kg. Later, the dosage was increased from 10 to 50 mg/kg for 7 days.

Experimental Design

Five groups of Zebrafish containing 10 animal each was used for this study. Group 1: Control, Group 2: CSE induced, Group 3: CSE induced + Treated with 5 mg/kg of quinine, Group 4: CSE induced + Treated with 10 mg/kg of quinine, and Group 5: CSE induced + Treated with 15 mg/kg of quinine. Gill inflammation was induced with CSE from 0.3 cig/L for 7 days. The treatment of quinine was given orally as for quinine treatment. 5, 10 and 15 mg/kg of quinine was given orally daily for 4 and 8 days. Fishes were euthanized and gill tissue was recovered from each group on day 4 and day 8 for biochemical and histopathological analysis and gene expression studies.

MPO activity Assay

Spectrophotometry assay of myeloperoxidase activity was carried out using an O-dianisidine dihydrochloride (o-dianisidine) solution. For extracting myeloperoxidase, gill tissue was homogenized at the ratio of 20mg/ml using potassium phosphate buffer. 14 µL of tissue homogenate was added into an Eppendorf tube. 400 µL of the O-dianisidine mixture was added to each tube and the absorbance was measured between 30 sec intervals up to 120 sec at 450 nm using a spectrophotometer. The MPO activity was expressed as units per mg of tissue.[10] The MPO activity was calculated using the following formula:

Absorbance [ΔA(t2-t1)]/Δmin x (1.13 x 10-2)/mg of tissue

NO Assay

The methodology described by Giustarini *et al.*[11] was used to estimate nitric oxide in zebrafish gill tissues. Breakdown of NO_3- (nitrite) to NO_2- was calorimetrically measured to estimate nitrate (NO). Griess reagent was used to determine NO_2-. Sulphanilamide (2%) and N-(1-naphthyl) Ethylenediamine (0.2%) was prepared with Phosphoric acid (5%) (v/v). 0.2 ml of gill tissue homogenate was mixed with 100 µl of Sulphanilamide (2%) and incubated for 5 mins at dark followed by the addition of 100 µl of N-(1-naphthyl) Ethylenediamine (0.2%). Absorbance was recorded at 540 nm and the nitric oxide concentration was expressed in µM/mL.

ROS Assay

The experimental procedure to estimate the reactive oxygen species (ROS) was adopted from Tunc *et al.*[12] with slight modification. Gill tissues 15 mg were washed using phosphate buffered saline to remove the adhered blood, blood clots and the extra muscles were carefully from the gill tissues. 0.1% NBT was added to the washed tissues and incubated for 45 min. This was centrifuged at 3000 rpm for 5 min and the suspended pellets were washed with 70% methanol twice and allowed to air dry. The dried pellet was immersed with 250µL of DMSO and 250 µL 2M KOH and vortexed for 20 sec. Then, the content was centrifuged at 3000 rpm for 5 mins. The supernatant was measured at 630 nm against DMSO/KOH mixture as blank. ROS was represented in percentage.

$$\% \text{ ROS} = (1 - A_1 / A_0) \times 100$$

A_1- Sample OD, A_0 – Control OD

Gene expression

DNA was extracted from treated and control group and subjected to PCR amplification for an assessment of gene expression. The isolated gills were rinsed in phosphate-buffered saline (ice-cold) to remove blood strain and blood clots. Gill tissue was ground using digestion buffer and incubated overnight at 55°C and subjected to DNA extraction by standard protocol. The air-dried pellet was

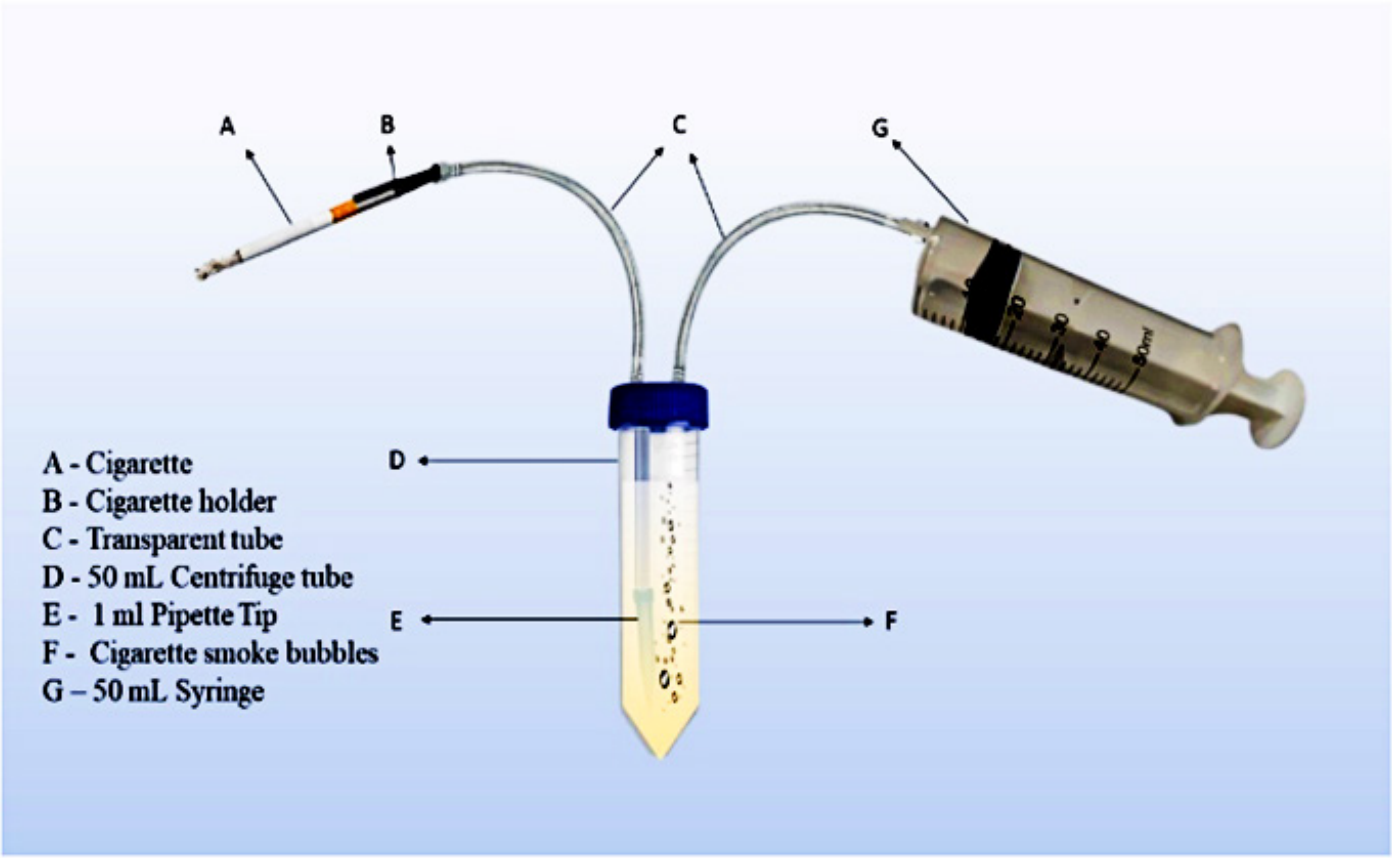


Figure 1: Cigarette smoke extracts setup.

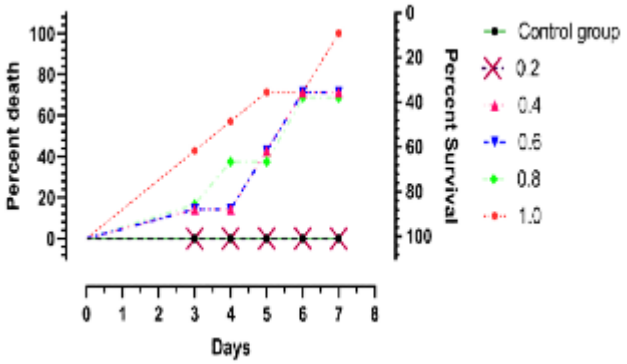


Figure 2: LC_{50} value. 0.3 cig/L was fixed based on the acute toxicity analysis.

dissolved in 50 µL of TE buffer and Thermo cycler gradient PCR was performed for IL-10 (For-5′ ACCAGCAGTTATTCGCACTCA 3′, Rev- 5′ TCAATCCAAAACACTTACGCATT 3′), IL-1 β (For-5′ TGTTTTCCTCCACAGAGCGT 3′, Rev - 5′ GGAGGAAGTGAAAACAGGGGA 3′) and β-Actin (For 5′ TCAAGGTGGGTGTCTTTCCTG 3′, Rev 5′ ATTTGCGGTGGA CGATGGAG 3′) primers are designed using NCBI Primer-BLAST tool.

Histopathology Studies

Histopathological analysis was carried out in the gill tissues to identify the infiltration of monocytes and changes in the gill tissue. Gill tissues were fixed in Bouin solution (15:5:1, picric acid: formaldehyde: acetic acid). Later to which, the tissues are rinsed with 70% ethanol for 15 mins and 4 times to remove the picric acid. Samples were dehydrated, fixed and embedded as described by Scheil *et al.*[13]

RESULTS

Determination of LC_{50} concentration of cigarette smoke extract

Figure 2 represents the LC_{50} determination of cigarette smoke extract (CSE). In order to determine the lethal concentration 50 (LC_{50}), 10 fishes were exposed to cigarette smoke extract at the concentration ranging from 0.2 to 1.0 cig/L. No toxicity was observed at 0.2 cig/L up to 7 days. However, dose-dependent toxicity was observed at concentrations from 0.4 to 1.0 cig/L. Increased mortality was observed at the highest tested concentration of 1.0 cig/L. Based on the obtained results in the acute toxicity analysis, 0.3 cig/L was fixed as LC_{50} concentration and this was further selected to induce gill inflammation based on LC_{50}.

Dose fixation for quinine treatment

Zebrafish were treated with a quinine concentration of 2 to 50 mg/kg. No mortality was observed in any of the tested doses. No

LC_{50} can be determined based on the nontoxic nature of quinine at the tested concentration. The oral dose range for the treatment of zebrafish after exposure to CSE was fixed as 5, 10 and 15 mg/kg body weight.

MPO Activity

MPO activity was measured as a marker for neutrophil activation. CSE has resulted in a significantly higher increase in MPO activity compared to the control group. No significant difference was observed in the 5 mg/kg body weight treated groups on the 4 days of treatment compared to the CSE-induced group. Dose dependent attenuation of MPO activity was observed in four- and eight-day quinine-treated zebrafish tissue with higher attenuation of MPO activity in the 15 mg/kg body weight groups (Figure 3A and 3B).

NO Levels

Significantly higher NO levels were observed in the CSE treated groups. Dose dependent decrease in the NO levels was observed in four- and eight-days quinine treated zebrafish tissue with higher attenuation of MPO activity in the 15 mg/Kg body weight groups (Figure 4A and 3B). The levels of NO were near normal at 15 mg/Kg body weight groups with no significant difference compared to CSE treated group (Figure 4A and 4B).

ROS Levels

The levels of reactive oxygen species (ROS) were presented in Figure 5A and 5B for 4 days and 8 days of quinine treatment. Increased levels of ROS were observed in the CSE treated group with a dose-dependent decrease in ROS levels at the quinine treated groups with the higher decrease at 15 mg/kg body weight group. Levels of ROS were much lower in 8 days treated group compared to the 4 days treatment group.

Histopathology

Inflammation in the gills, damaged gill filaments and accumulation of neutrophils were observed in the CSE treated Zebrafish, whereas the intensity of the inflammation and neutrophil accumulation was tended to be decreased in the 5, 10 and 15 mg/kg quine treated groups. A clear restoration of gill structure was observed with 10 and 15 mg/kg treated group, with much clear observation at 15 mg/kg quinone treated group (Figure 6).

Pro and anti-inflammatory gene expression

The levels of proinflammatory (IL-1b) and anit-inflammatory (IL-10) cytokine levels were assessed during the CSE treated group and quinine treated group. Gene expression levels of the pro-inflammatory cytokine, IL-1b was found to be higher in the

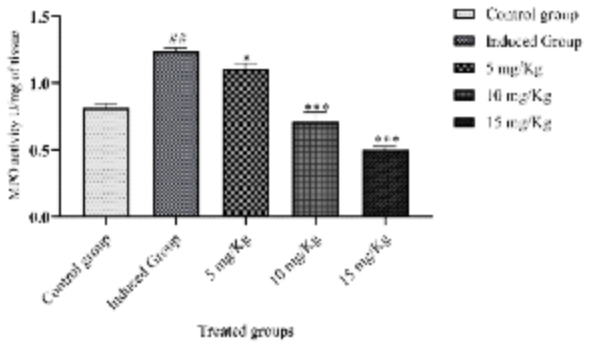


Figure 3 A: #*p*< 0.05 vs the control group, ∗*p*< 0.05 vs the CSE group.

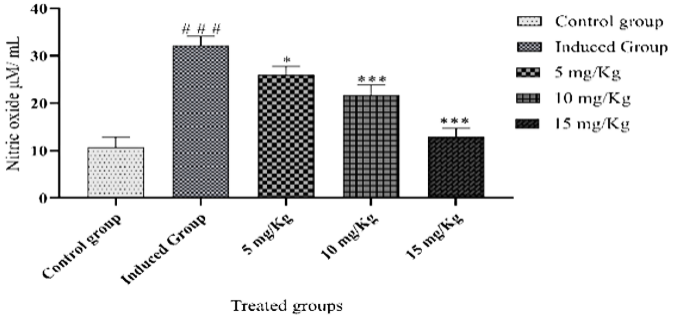


Figure 4 A: #*p*< 0.05 vs the control group, ∗*p*< 0.05 vs the CSE group.

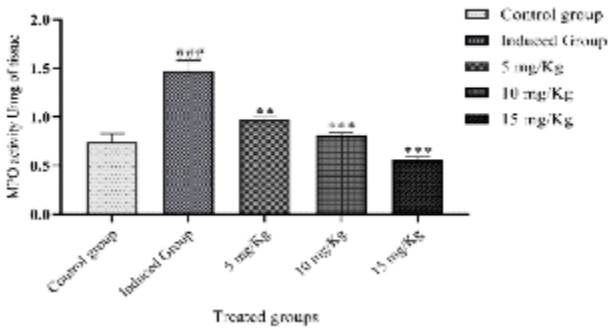


Figure 3 B: #*p*< 0.05 vs the control group, ∗*p*< 0.05 vs the CSE group.

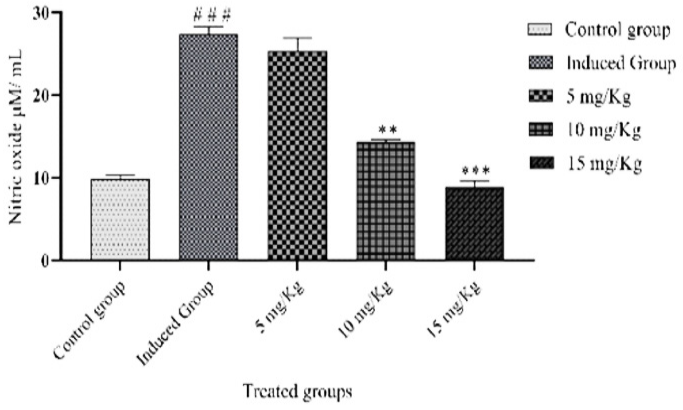


Figure 4 B: #*p*< 0.05 vs the control group, ∗*p*< 0.05 vs the CSE group.

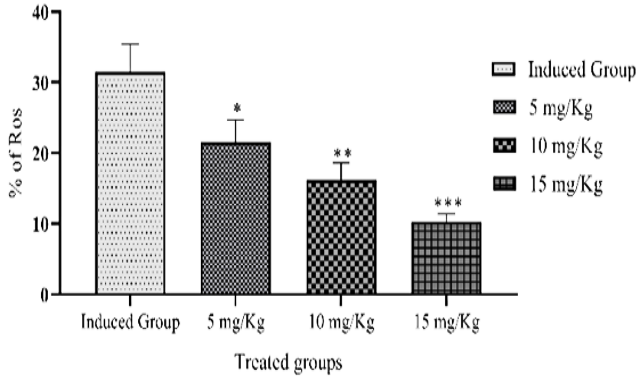


Figure 5 A: *p< 0.05 vs the CSE group.

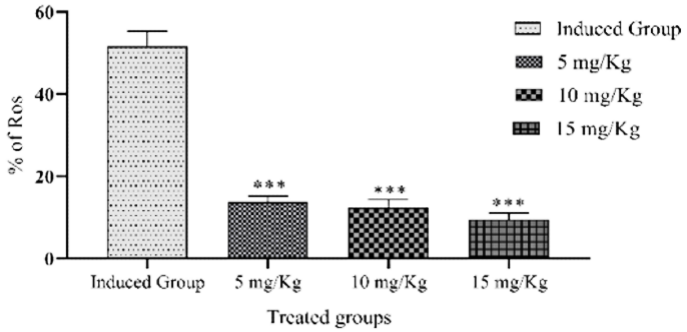


Figure 5 B: *p< 0.05 vs the CSE group.

CSE treated group and it gradually decreased with the increase in the treatment concentration of quinine from 5, 10 and 15 mg/kg treated group. The expression level of anti-inflammatory cytokine IL-10 was found to be in the increasing trend with 5, 10 and 15 mg/kg body weight treated group (Figure 7).

DISCUSSION

Ameliorative effect of quinone against cigarette smoke induced gill inflammation was tested in Zebrafish model at the concentration of 5, 10 and 15 mg/kg body weight. Prior to experimental procedure and IC_{50} value of cigarette smoke extract was identified using different concentration of cigarette smoke extract. As indicated by the results no cytotoxic effect was observed at the concentration of 0.2 cig/L, whereas toxicity response was observed from the concentration of 0.4 cig/L. Based on this observer the IC_{50} value of CSE was identified at the concentration of 0.3 cig/L which is used for further experimental studies. In recent days Zebrafish is gaining much attention in accessing the toxicity of drugs, screening of candidate molecules and to study disease mechanisms due to their smaller size and transparent in nature. The smaller size makes the experiment feasible within small lab space and small volume of test article.[7]

A dose ranging experiment with quinine at 2 to 50 mg/kg indicates that Zebrafish is well tolerated even up to the highest level of

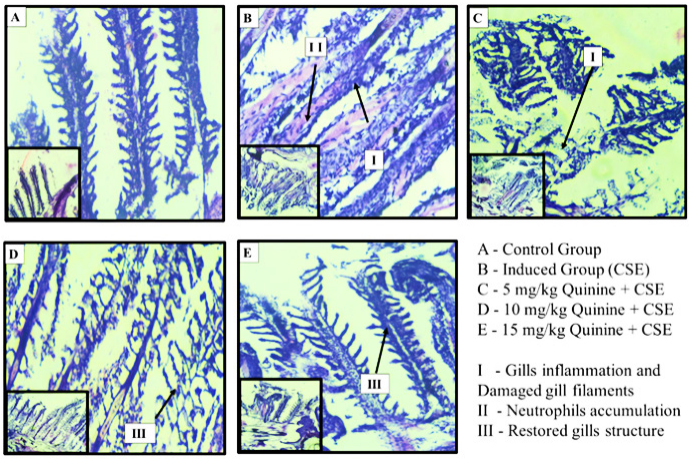
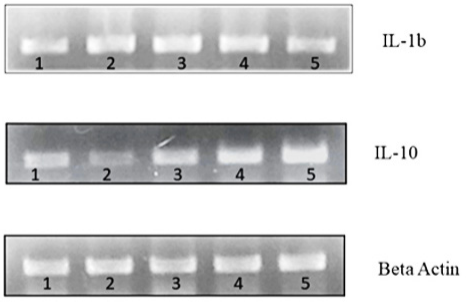


Figure 6: Histopathology studies. In the induced group, was an accumulation of neutrophils along with gill inflammation and injured filaments. Treatment groups reduced inflammation dose dependent manner.



Lane 1 – Control Group, **Lane 2** – induced group (CSE), **Lane 3** – 5 mg/kg quinine +CSE, **Lane 4** – 10 mg/kg quinine + CSE, **Lane 5** – 15 mg/kg quinine + CSE

Figure 7: Gene expression studies.

quinine tested, 50mg/kg with no mortality. For this study, 5 mg, 10 mg and 15 mg was selected as experimental concentration of quinine to be tested for its activity against cigarette smoke induced gill inflammation.

Quinine has showed its ameliorating property against CSE by the ability to reduce myeloperoxidase activity in a dose and time dependent manner with higher inhibition of MPO activity at 15mg/kg treated animals for 8 days. Myeloperoxidase, prominent enzyme presents in which plays a very important role during host defence system and inflammation. Degranulation of neutrophil either by pro-inflammatory conditions of the by the presence of reactive oxygen species will tend to release myeloperoxidase, which act as a bio-marker of inflammation.[14] It was previously reported that the quinine can inhibit the myeloperoxidase via competitive inhibition and thus server as immune suppressor.[15] In the current study, we have demonstrated that the myeloperoxidase activity was inhibited by the quinine thus ameliorating the effects of CSE by reducing the inflammation.

Nitric oxide is reported as one of the major contributing factors for the inflammation and injury of the lungs when induced by toxicants.[16] Besides exhibiting MPO inhibitory activity, our

results has also estimated that quinine exhibited a dose dependent decrease in the levels of NO production in the CSE induced and quinine treated groups. The values of MPO activity and NO were near normal after treatment with quinine for 8 days at 15 mg/kg. Thus, is evident that the quinine reduced the toxic effects of inflammation by reducing the nitric oxide levels as observed in the in the CSE + quinine treated group. Both these MPO activity and NO results indicate that quinine can prevent from the toxic effects of CSE. Involvement of xanthine oxidase activity as a source of uric acid and reactive oxygen species leading to the pro-oxidative and pro-inflammatory effects during pathological conditions was also reported.[17] High level of uric acid can also be a source to induce oxidative stress. Considering this, we have also estimated the levels of ROS, nitric oxide.

Cigarette smoke has been reported to increase the oxidative stress leading to the dysregulation of reactive oxygen species production as the marker of oxidative stress.[18] Due to the imbalance in the generation and scavenging of ROS, the ROS accumulates leading to tissue damage.[19] Quinine has been reported to be involved in the electron transport chain and decreasing its activity.[20] ROS estimation showed a marked increase in the ROS levels in the CSE induced group, whereas the quinine treated groups showed dose-dependent decrease in the ROS levels with low levels of ROS production in the 15mg/kg treated group. The observed results were consistent with the levels of MPO and NO. Quinine inhibited ROS production and oxidative stress by radical scavenging effects which are the possible mechanisms of the anti-inflammatory activity. Neutrophils and macrophages generate pro-inflammatory mediators, including IL-1beta and IL-6 in lung inflammation.[21] In this study, an decrease in IL-1b beta revealed that quinine plays a pro-inflammatory role, while an increase in IL-10 showed an anti-inflammatory response.[22] CS exposure is associated with higher MPO activity and increases in the proinflammatory cytokine IL-1beta.[23] Our studies found that quinine attenuated MPO activity which is related to levels of IL-1beta in the zebrafish in a dose-dependent manner similar to anti-inflammatory potential of potato extract which significantly reduced IL-10 expression level after CS exposure. Naringin treatment also reduced IL-10 expression level significantly in dose dependent manner for l and 8 weeks of CS exposure.[24]

In our study gill inflammation and damaged gill filaments were observed in the CSE treated group and the effects were decreased in the quinine treated group. It was previously reported that the increase in inflammation will lead to an increased accumulation of neutrophils and lymphocytes at the lamellae of the gills leading to damage to gill filaments.[25] The results of our study are inconsistent with the previous report on respiratory immunology, where gill inflammation and damage to gill filaments were observed in the CSE-treated group.

CONCLUSION

The results obtained in our study it is obvious that quinine has the potential to decrease the toxic effects of CSE by combating inflammation, reducing the ROS, MPO, NO and pro-inflammatory cytokine levels and increasing the anti-inflammatory cytokine levels. The present study has shown that quinine ameliorates the cigarette smoke extract induced inflammatory changes in Zebrafish model.

CONFLICT OF INTEREST

The author declares no conflict of interest.

ABBREVIATIONS

COPD: Chronic obstructive pulmonary disease; **CSE:** Cigarette smoke extracts; **IL-1β:** Interleukin 1 beta; **IL-10:** Interleukin 10; **ROS:** Reactive oxygen species; **MPO:** Myeloperoxidase; **NO:** Nitric oxide: Lipid peroxidation; **DMSO:** Dimethyl sulfoxide; **KOH:** Potassium hydroxide; **Cig:** Cigarette; **PCR:** Polymerase chain reaction.

SUMMARY

- Quinine, an alkaloid, was analysed for its efficacy in reducing oxidative stress and inflammation of cigarette smoke extracts in Zebrafish.
- Zebrafish exposed to cigarette smoke extracts induced oxidative stress, Reactive Oxygen Species (ROS), Lipid peroxidation (LPO), and Myeloperoxidase (MPO) gradually increased.
- In CSE treated group, Inflammatory mediators IL-1β significantly increased and IL-10 significantly decreased.
- On treating the CSE-induced zebrafish with quinine, the oxidative stress markers reduced in dose-dependent manner. Inflammatory marker, IL-1β decreased and IL-10 increased in dose-dependent manner.
- Histopathological studies showed restoration of gill structure on treatment with quinine which was damaged by exposure to CSE.
- The bioactive compound of quinine ameliorates CSE-induced oxidative stress and inflammatory mediators.

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