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In vivo effect of chronic nicotine exposure on outcome of *Plasmodium berghei* ANKA malaria

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ABSTRACT

Objective: To assess effect of nicotine, major addictive component of tobacco smoke, on outcomes of the deadly malaria parasite using mice as animal model.
Methods: Male Swiss albino mice were treated with 100 and 200 μg/mL of nicotine in drinking water daily for 6 weeks followed by *Plasmodium berghei* ANKA (PbA) infection. On the seventh day of post infection (p.i.), physical, clinical, histopathological, biochemical and hematological parameters were assessed. Data were analyzed using SPSS software.
Results: Nicotine was significantly ($P < 0.05$) positively associated with lower levels of hemoglobin (Hb), hematocrit (HCT), red blood cells (RBCs), C-reactive protein (CRP) and uric acid (UA), higher risk to incidence of pulmonary edema, elevated level of liver and kidney biomarkers. Also significant increment ($P < 0.01$) of monocyte-lymphocyte count ratio (MLCR) was observed. Risk to high temperature, lower platelet count, high parastemia and cerebral malaria was lesser in mice treated with nicotine (100 and 200 μg/mL) followed by PbA infection than the positive control. Lack of neurological symptoms might be accounted to the anti-inflammatory property of nicotine that could inhibit production of pro-inflammatory mediators responsible for occurrence of cerebral malaria.
Conclusions: This study showed that despite down regulation of most cerebral malaria symptoms nicotine was strongly associated with increased risk to most clinical symptoms of malaria. Thus, like in respiratory infections, nicotine use might enhance susceptibility to malaria.

1. Introduction

Despite its adverse health impacts and a leading cause of death, tobacco smoke has been used by millions globally[1]. Worldwide, about 30% of adult males are estimated to smoke tobacco[2,3]. Tobacco smoke is responsible for fatal non-communicable disorders such as cancers of vital organs and mortality[4]. Moreover, it affects circulatory, respiratory, and reproductive systems; causes stroke, chronic obstructive lung diseases, bronchitis, and infertility[5,6]. Despite the widely publicized harmful effects of smoking, most smokers return to the habit after short time of quitting[7].

Although tobacco contains a large variety of substances, nicotine, which acts at nicotinic acetylcholine receptors (nAChRs) in the central and peripheral nervous systems, is considered as the major addictive component[8]. The main route of exposure to nicotine is through inhalation of the tobacco smoke. In mice, it is readily absorbed via respiratory tissues, skin, and the gastrointestinal tract, and then enters blood stream and reaches the brain within a minute after exposure[9].

Epidemiological and experimental studies have shown that, exposure to tobacco and nicotine increases susceptibility to respiratory tract infections[10]. Tobacco smokes increase risk to infectious diseases by inducing physiological and structural alteration on target sites in human, or increase pathogen virulence feature, or through dysfunctioning of immune system[10]. As respiratory system is the primary site of interaction with the tobacco smoke, any undesirable outcomes associated to tobacco or its constituents could affect the outcome of respiratory infections. However, some components of tobacco smoke such as nicotine can crosses blood brain barrier (BBB) and reach brain within few seconds[11]. Its probability of concentration in blood at a single exposure and effects might be insignificant. But heavy smokers can easily maintain the optimum effective dose of nicotine through its rapid intake since smokers has to adjust their smoking behavior

to maintain a specific nicotine concentration throughout the day[12-14]. Thus, effect of nicotine on blood pathogens could be as equally important as respiratory infections, since longer exposure of the blood pathogen to the nicotine could be possible. To the authors' knowledge, there is no report on the effect of nicotine available in blood on blood parasites. Accordingly, this study was designed to assess *in vivo* effect of nicotine on outcome of one of the deadly blood parasites, malaria infection, using Swiss albino mice as animal model.

2. Materials and methods

2.1. Experimental animal

Male Swiss albino mice (aged 6–8 weeks and weighing 20–30g) were housed in transparent plastic cage having SS sipper 250 mL water bottle. Wood shaving was used as bedding and it was replaced every morning after cleaning and disinfecting the cage with 70% alcohol. The mice were kept under unlimited access to commercial pellet food and fresh tap water *ad libitum*.

2.2. Nicotine repeated toxicity test

Sixteen male Swiss albino mice were randomly divided into two groups ($n = 8$ per group). After fasting for a period of 2 h[15,16], mice in the first group were treated with 100 μg/mL and the second group with 200 μg/mL of nicotine [nicotine hydrogen tartrate salt (Sigma–Aldrich)] in drinking water, and observed for any signs of toxicity daily for 2 weeks. Mice were checked for any observable signs of toxicity[15].

2.3. Mice grouping and treatment

After a week adaptation period in laboratory, mice ($n = 48$) were grouped into six categories (I–VI), eight per group. The first two groups (I–II) were treated with 100 μg/mL of nicotine, while the second two (III–IV) were treated with 200 μg/mL nicotine in drinking water daily for 6 weeks following a procedure used by Lai *et al.*[17]. For all the treatments, nicotine was freshly prepared every day and kept under dark condition in especial bottle covered with aluminum foil to avoid its oxidation. Nicotine treatment was started at an initial concentration of 25 μg/mL, and then increased to 50 μg/mL on Day 3 and 100 μg/mL on Days 5. For the other treatment group final concentration of 200 μg/mL was adjusted on Day 8[17]. Mice in the other two groups (V and VI) were used as positive and negative control respectively, and were only exposed to normal water for the same duration to the nicotine treated groups. In the next step, as indicated in the following section, one of the final two groups was infected with PbA to be used as positive control. As treatment duration is lengthy (6 weeks) and we were dealing with toxic chemical nicotine, thus to minimize the probability of losing data from all mice and to get at least optimum sample for further analysis, number of mice included in each group ($n = 8$) exceed the common number ($n = 6$) mostly used. Food and water intake of all mice in the experiment was daily monitored.

2.4. Parasite preparation and mice infection

PbA parasite was obtained from donor mice carrying 30% of parasitemia. After the mice was terminally anesthetized using diethyl ether and exposure in a closed container, through cardiac puncture, infected blood was collected and then diluted to 1×10^5 PRBCs in PBS. Each mouse from the first and second groups (I and III, treated with 100 and 200 μg/mL nicotine in drinking water, respectively) and

positive control (V) were infected with 100 μL of the diluted PRBCs through intra-peritoneal (*i.p.*) injection on the last day of the 6th week of the treatment period. Briefly, the experimental mice were divided into groups: (1) uninfected (negative control), (2) uninfected but received nicotine (100 and 200 μg/mL) in drinking water for 6 weeks, (3) only PbA infected (positive control), (4) PbA infected and received nicotine (100 and 200 μg/mL) in drinking water for 6 weeks.

Before the actual experimentation was commenced, survival time of Swiss albino mice infected with PbA (1×10^5 PRBCs in PBS) was checked. On the seventh day of p.i., rectal temperature was measured using digital thermometer, body weight was taken, few drops of blood samples from tail snip were used for measurement of blood glucose (Glu) level [(Hemocue® Glucose 201 analyzer, Angelholm, Sweden)] and hemoglobin (Hb) levels [(Hb analyzer, Hemocue™ haemoglobinometer, Angelholm, Sweden)].

Parasite load was determined in blood smears (thin and thick) having stained in 10% Giemsa for 10 min. Percent (%) parasite load was calculated by dividing infected RBCs to total RBCs and multiplied by 100. Parasite load of nicotine treated mice was compared with the positive control.

2.5. Assessment of cerebral syndromes

Incidence of clinical signs of cerebral malaria (CM) that involves neurological syndromes such as ataxia, paralysis, deviation of the head, convulsions, decrease in body temperature, loss of vascular cell integrity, tissue edema, hemorrhages in the brain of mice, and congestion of micro-vessels with parasitized erythrocytes and/or mononuclear cells was assessed[18,19].

After clinical and physical conditions were assessed, each mouse was terminally anesthetized, and blood sample was collected through cardiac puncture for hematological and biochemical tests. Furthermore, brain was carefully removed, weighed and processed for histopathological study. Liver, kidney, thymus and spleen were used for determination of relative organs weight. Likewise, lungs were removed and used for assessment of status of pulmonary edema.

2.6. Effect of nicotine on hematological parameters, kidney and liver functions

Blood samples were collected from the mice in EDTA coated tube. The blood samples were used for quantification of total white blood cells (WBCs), lymphocytes, red blood cells (RBCs), hematocrit (HCT), and platelets using CBC machine [Automated complete blood cells (CBC) Analyzer: Sysmex KX-21]. Some portion of the blood samples, collected in EDTA coated tube, was centrifuged (Centrifuge 4515R) at 10000 r/min for 10 min to separate serum. The supernatant was transferred into new Eppendorf tube followed by immediate measurement of liver enzymes, serum glutamic oxaloacetate transaminase (sGOT), serum glutamic pyruvic transaminase (sGPT), albumin (Alb), biomarkers of kidney functions such as creatinine (Cr) and urea, and inflammation indicators, uric acid (UA) and C-reactive protein (CRP) were carried out using automated immunochemical analyzer (Axsym MEIA 3rd Generation).

2.7. Body and relative organs weight

Body weight of each mouse, nicotine treated and the control groups were monitored over 6 weeks. Relative weight of thymus and spleen, liver and kidney were measured. Relative organ weight was calculated by dividing weight of each organ to body weight of the mice and then

multiplied by 100.

2.8. Histopathological analysis

For histological analysis, brains of sacrificed mice were collected in 10% buffered neutral formaldehyde. Paraffin-embedded brain tissue was sectioned, and stained with hematoxylin and eosin. Slides were coded and scored blind for histological evidence of cerebral syndromes and liver and kidney damage.

2.9. Pulmonary edema

Pulmonary edema status was assessed in lungs of nicotine treated mice prior to PbA infection and the positive control. Briefly, the wet weight of lung from terminally anesthetized mice was measured right away after its removal and the dry weight was determined after overnight incubation at 80 °C. Then the ratio of wet to dry weight was calculated to determine pulmonary edema state.

2.10. Data analysis

Data were checked for their completeness and then analyzed using SPSS software (version 20). Data were expressed in mean ± SEM. One-way ANOVA, followed by Turkey's HSD *post-hoc* test was used to compare the effect of nicotine on different variables. Values of $P < 0.05$ were considered statistically significant.

2.11. Ethical consideration

The study was ethically approved by Research and Ethical Board of College of Health Science, Jimma University, Ethiopia. All procedures that involved mice were handled in humane way and extreme precautions were taken to avoid stress induced by poor handling techniques. On scientific and ethical grounds, blood samples were collected from mice under final anaesthesia on the last day of survival time. However, in the preliminary study on survival time to decide on the cutoff point for the follow-up and treatment of mice, mice were dead without euthanasia, due to the experimental intervention.

3. Results

In the assessment made to estimate the survival time of mice infected with 1×10^5 PRBCs in PBS, the mean survival time was found 7.5 (6–9) days. Thus, with close observation, the seventh day of p.i. was considered as cutoff point for follow-up, and treatment was extended to Day 7 of p.i.

3.1. Repeated toxicity study

The toxicity study showed that the chosen concentration of nicotine (100 and 200 μg/mL) did not cause mortality within the first 24 h and the following 2 weeks. Physical and behavioural observations of the experimental mice also indicated no visible signs of overt.

3.2. Food and water intake

During the study period, mean food intake of each mouse per cage on daily basis showed gradual significant reduction ($P < 0.05$) in mice that received 200 μg/mL of nicotine as compared to the negative control. Also mice treated with both concentration of nicotine (100 and 200 μg/mL) showed significant reduction ($P < 0.01$) of water intake as early as the first week of the follow-up, while the control groups consumed an average of 7.2 mL of water without nicotine per mouse daily (Figure 1).

3.3. Effect of nicotine on body and organ weights

At intial stage of the nicotine treatment (week 1–3), body weight of all mice showed a sort of an increment, but gradually as treatment duration longer, from week 4 to 6, significant weight reduction ($P < 0.05$) was observed in mice treated with 200 μg/mL of nicotine (Figure 2).

Even though there were an increasing patterns in liver and kidney weights and decreasing patterns in the weight of spleen and thymus, significant differences ($P > 0.05$) in kidney and spleen weight were
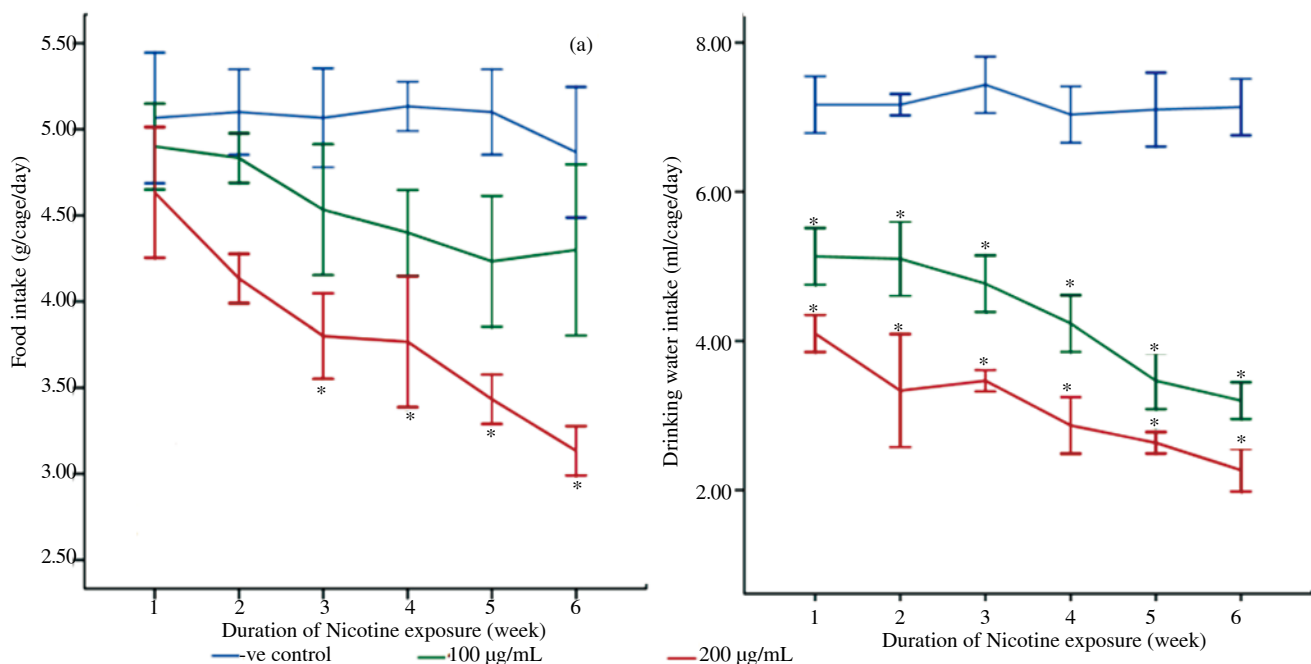

Figure 1. Food consumption (a), and drinking water intake (b) (mean ± SEM) of male Swiss albino mice (*n* = 8) during administration of nicotine in drinking water. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of negative control.

not observed between nicotine treated and the negative control mice. However, weight of liver and thymus of mice treated with 200 μg/mL of nicotine was significantly different ($P < 0.05$) from the negative control (Figure 3).
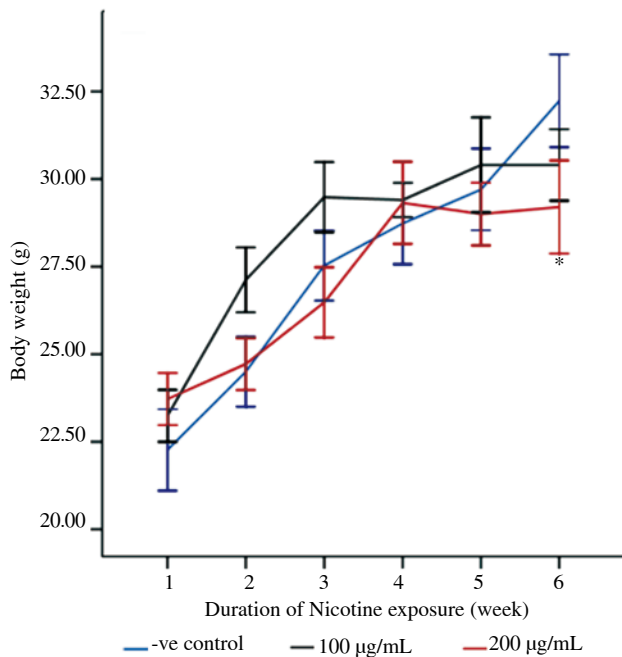

Figure 2. Body weight (mean ± SEM) of Swiss albino mice ($n = 8$) treated with nicotine in drinking water daily for 6 weeks and negative control. Value with asterisk is significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of negative control.

3.4. Effect of Nicotine on outcomes of malaria infection

According to assessment made on the 7th day of PbA p.i., comparable parasite load was observed in mice treated with nicotine (100 μg/mL) prior to PbA (20.87%) and positive control (23.80%). But significant parasite load reduction ($P < 0.01$) was observed

in mice treated with 200 μg/mL of nicotine (14.98%) prior to the infection. Although there was decreasing pattern of glucose level, and increasing patterns in rectal temperature on the 7th day of p.i., significant differences were not observed ($P > 0.05$) between nicotine treated prior to PbA infection and the positive control mice. However, nicotine (both 100 and 200 μg/mL) showed significant effect ($P < 0.05$) on the level of Hb in mice exposed to nicotine prior to PbA infection (Figure 4).

Likewise nicotine of the chosen concentration was positively associated with incidence of pulmonary edema. This was observed by significant reduction ($P < 0.01$) of pulmonary edema status in mice treated with higher dose of nicotine (200 μg/mL), prior to PbA infection than the positive control (Figure 5).

3.5. Nicotine induced hematological and biochemical changes

Data from mice treated with nicotine suggested that, although it has no toxicity, nicotine at higher concentration (200 μg/mL) had significant effect ($P < 0.05$) on counts of WBC, RBC, HCT, Hb and lymphocyte as compared to the negative control mice. Likewise, during PbA infection, higher concentration of nicotine had shown negative effect on the levels of some hematological parameters. At concentration of 200 μg/mL, nicotine was associated with significant reduction ($P < 0.05$) in the counts of WBC and RBC besides similar effect in levels of HCT and Hb in mice treated with nicotine followed by PbA infection as compared to only the positive control. Hb concentration was significantly reduced ($P < 0.05$) even in mice treated with lower concentration (100 μg/mL) of nicotine. The other hematological parameters (lymphocytes and platelet count) didn't show significant differences ($P > 0.05$) between nicotine treated and control groups (Table 1).

There was significant difference in the monocyte-lymphocyte count ratios (MLCR) distribution between the control and nicotine treated mice. MLCR was significantly increased ($P < 0.01$) in mice treated with 200 μg/mL concentration than control. But neutrophil-lymphocyte count ratios (NLCR) didn't show significant differences
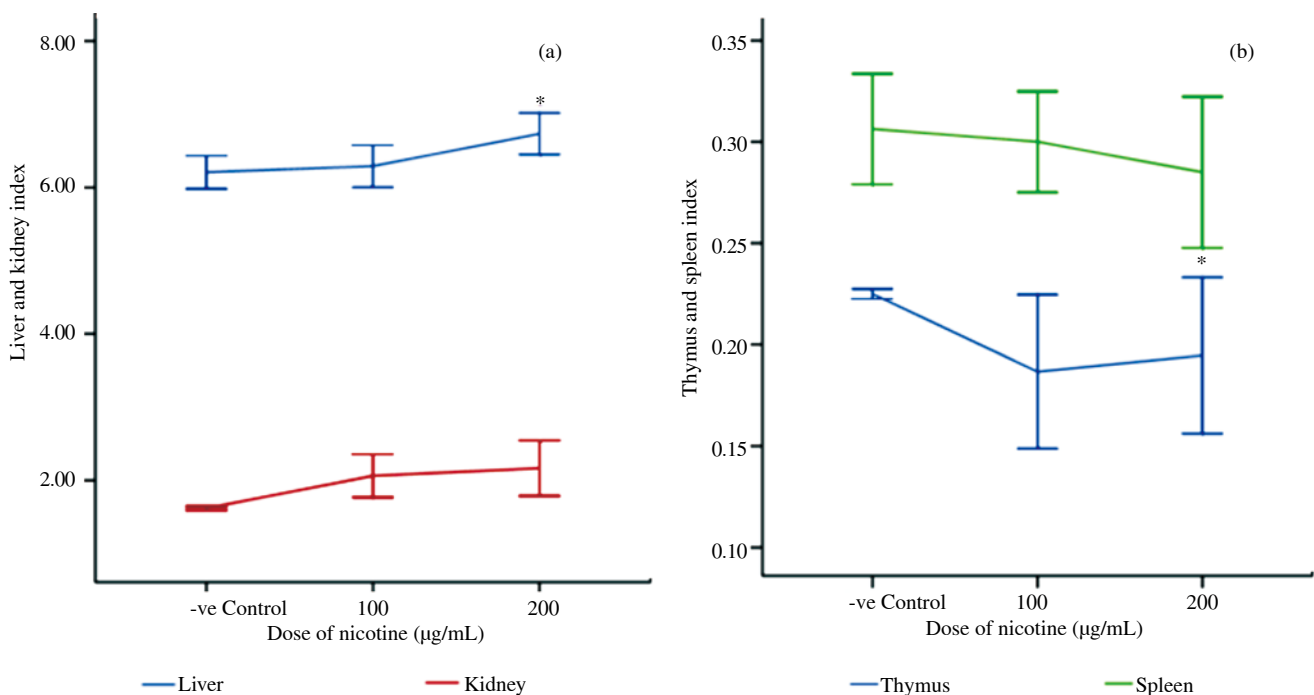

Figure 3. Relative organs weight (mean ± SEM) of liver and kidney (a), and spleen and thymus (b) of mice ($n = 8$) treated with nicotine in drinking water daily for 6 weeks and control. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of negative control.
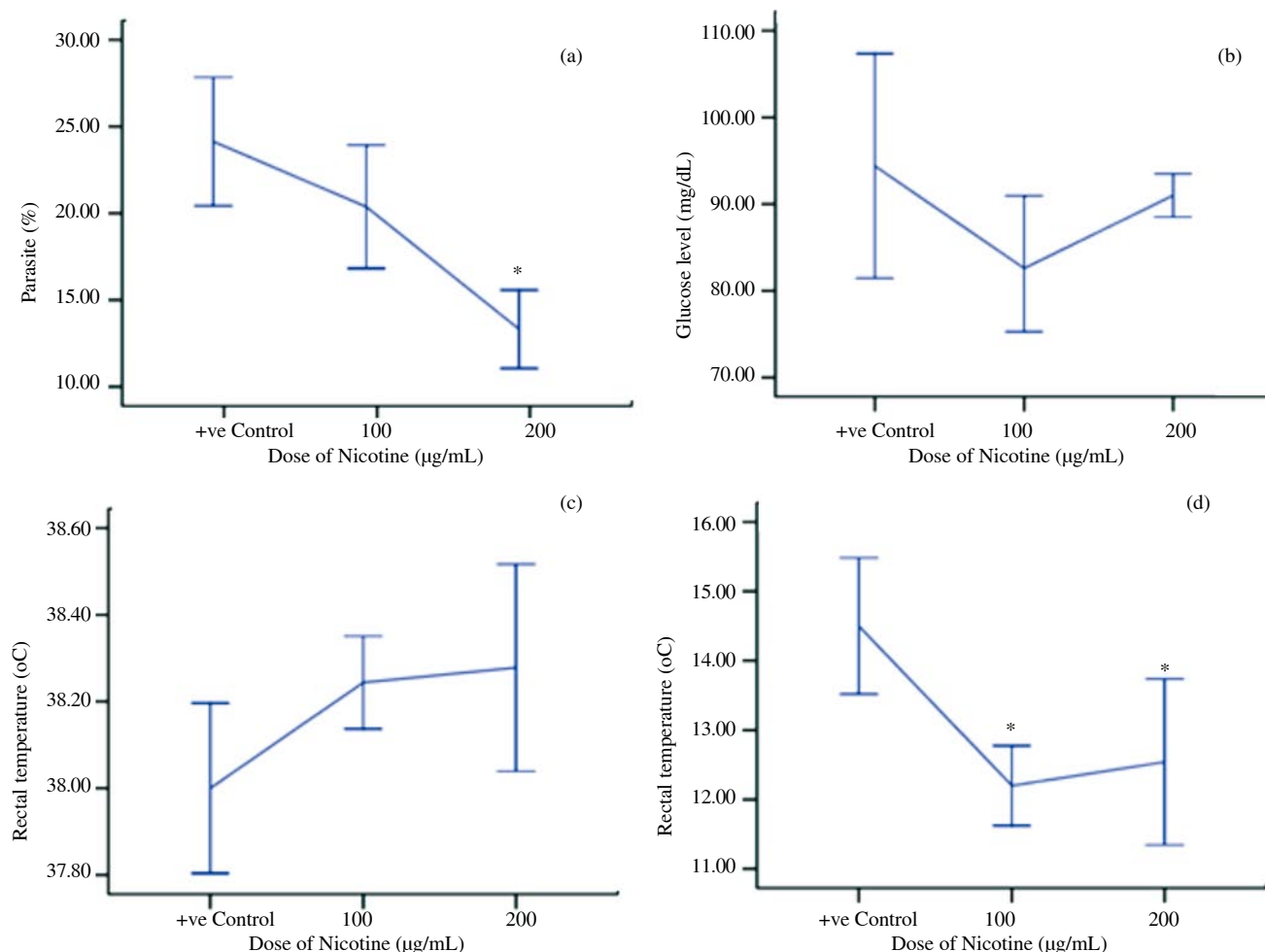
Figure 4. Clinical conditions; Parasite load (a), glucose level (b), rectal temperature (c), hemoglobin level (d) and of Swiss albino mice (*n* = 8) treated with nicotine in drinking water daily for 6 weeks prior to PbA infection on day 7 p.i. and positive control.
Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of positive control.

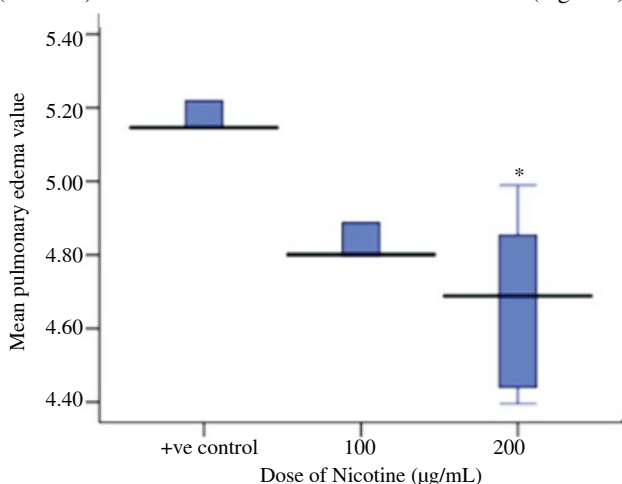($P > 0.05$) between nicotine treated and the control mice (Figure 6).

Figure 5. Pulmonary edema status (mean ± SEM) of Swiss albino mice (*n* = 8) treated with nicotine in drinking water daily for 6 weeks prior to PbA infection. Value with asterisk is significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of positive control.

Although rise of liver and kidney biomarkers are common symptoms during malaria infection, liver function biomarkers was found significantly different ($P < 0.05$) between nicotine treated followed by PbA infection and the positive control. This is in mice treated with nicotine followed by PbA infection, liver enzymes,

sGOT and sGPT were significantly elevated ($P < 0.01$) at higher concentration (200 µg/mL), and also at 100 µg/mL of nicotine concentration for sGPT than positive control group. Level of sGPT in nicotine treated mice (200 µg/mL) was greater than three times (3×) the level of sGPT measured in positive control mice. Similarly, level of albumin was significantly reduced ($P < 0.05$) in mice treated with 200 µg/mL of nicotine followed by PbA infection than control. Kidney function indicator, creatinine, was significantly elevated ($P < 0.001$) in mice treated with 100 and 200 µg/mL of nicotine followed by PbA infection than positive control, but level of urea was significantly affected ($P < 0.01$) only at higher concentration of nicotine (200 µg/mL). Similar pattern of liver and kidney biomarkers level was observed in mice treated with nicotine for 6 weeks daily without PbA infection when compared to the negative control (Table 2).

Moreover, the inflammatory biomarkers analyzed showed that there was a reduction pattern in level of this biomarkers in mice treated with nicotine followed by PbA infection. This is level of CRP and UA was significantly reduced ($P < 0.05$) in mice treated with 100 and 200 µg/mL of nicotine in the drinking water (Figure 7).

3.6. Incidence of severe malaria symptoms

Brain tissue of mice treated with both concentration of nicotine (100 and 200 µg/mL) prior to PbA infection was found normal (Figure 8). On the seventh day of p.i., all mice treated with
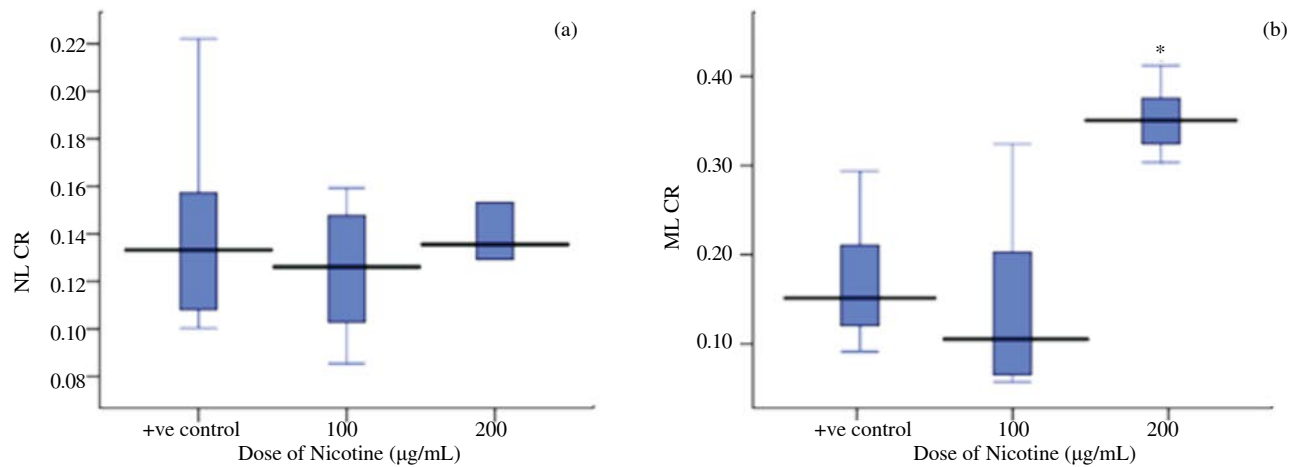
Figure 6. Box plots showing distribution of neutrophil-lymphocyte count ratios (NLCR) (a) and monocyte-lymphocyte count ratios (MLCR) (b) in Swiss albino mice (*n* = 8) treated with nicotine in drinking water daily for 6 weeks prior to by PbA infection. Value with asterisk is significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the positive control.

Table 1

Hematological tests in mice (mean ± SEM) chronically treated with nicotine prior to PbA infection and control.

Group	WBC (×10³/µL)	RBC (×10⁶/µL)	Hb (g/dL)	HCT (%)	Plt (×10³/µL)	Lym (%)
-ve control	3.90 ± 1.01	8.07 ± 0.74	17.70 ± 1.15	44.30 ± 1.10	709.00 ± 34.90	87.20 ± 0.56
100 µg/mL§	3.67 ± 1.25	8.10 ± 1.01	13.90 ± 0.67*	39.10 ± 0.60	722.00 ± 27.00	78.00 ± 1.90
200 µg/mL§	3.49 ± 1.03*	7.04 ± 0.98*	12.30 ± 0.30**	36.20 ± 0.50*	636.00 ± 24.00	67.00 ± 1.10*
+ve Control	6.64 ± 1.04	7.89 ± 1.14	14.14 ± 0.94	39.44 ± 0.20	353.00 ± 38.00	74.92 ± 1.40
100 µg/mL + PbA§§	5.84 ± 0.84	6.59 ± 0.80	11.82 ± 1.47*	32.36 ± 1.30	332.00 ± 17.00	73.76 ± 1.80
200 µg/mL + PbA§§	3.60 ± 0.62*	6.46 ± 0.74*	11.54 ± 1.37*	32.62 ± 5.10*	369.00 ± 32.00	77.74 ± 1.20

NB: Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of control (*: Significant difference at $P < 0.05$, **: at $P < 0.01$). Significance level of uninfected but nicotine treated (§) was compared with -ve control group, but nicotine treated and PbA infected (§§) were compared with +ve control group. Lym: Lymphocyte; Plt: Platelet.

Table 2

Liver and kidney biomarkers profile (mean ± SEM) in Swiss albino mice (*n* = 8) treated with nicotine daily for 6 weeks prior to PbA infection.

Group	GPT (IU/L)	GOT (IU/L)	Urea (mg/dL)	Cr (mg/dL)	Albumin (g/dL)
Negative control	11.36 ± 2.10	10.52 ± 3.45	18.05 ± 1.90	0.210 ± 0.033	3.17 ± 0.10
100 µg/mL§	54.00 ± 20.30**	53.00 ± 17.90**	25.37 ± 3.60	0.310 ± 0.041*	2.57 ± 0.11**
200 µg/mL§	124.00 ± 10.10**	85.00 ± 22.50**	31.00 ± 8.10*	0.320 ± 0.012*	2.64 ± 0.09**
Positive control	127.00 ± 15.50	257.00 ± 38.20	40.91 ± 4.70	0.360 ± 0.047	2.06 ± 0.12
100 µg/ml + PbA§§	173.00 ± 18.70**	318.00 ± 44.60	46.33 ± 3.07	0.300 ± 0.000***	1.75 ± 0.10
200 µg/ml + PbA§§	398.00 ± 25.20**	388.00 ± 39.50**	50.67 ± 3.78**	0.300 ± 0.000***	1.51 ± 0.12*

NB: Significance level of uninfected but nicotine treated (§) was compared with negative control group, but treated and infected with PbA (§§) was compared with positive control group. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls (*: Significant different at $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Figure 7. Level of C-reactive protein (CRP) (a) and uric acid (UA) (b) in Swiss albino mice treated with different concentration of nicotine in drinking water daily for 6weeks prior to PbA infection. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls (*: Significant different at $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).
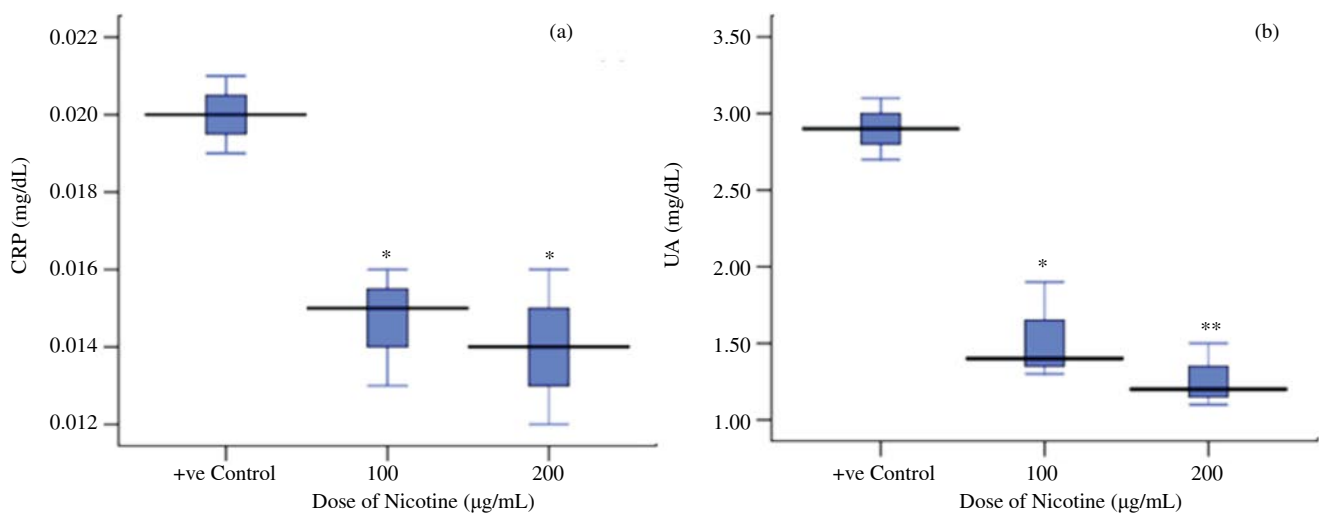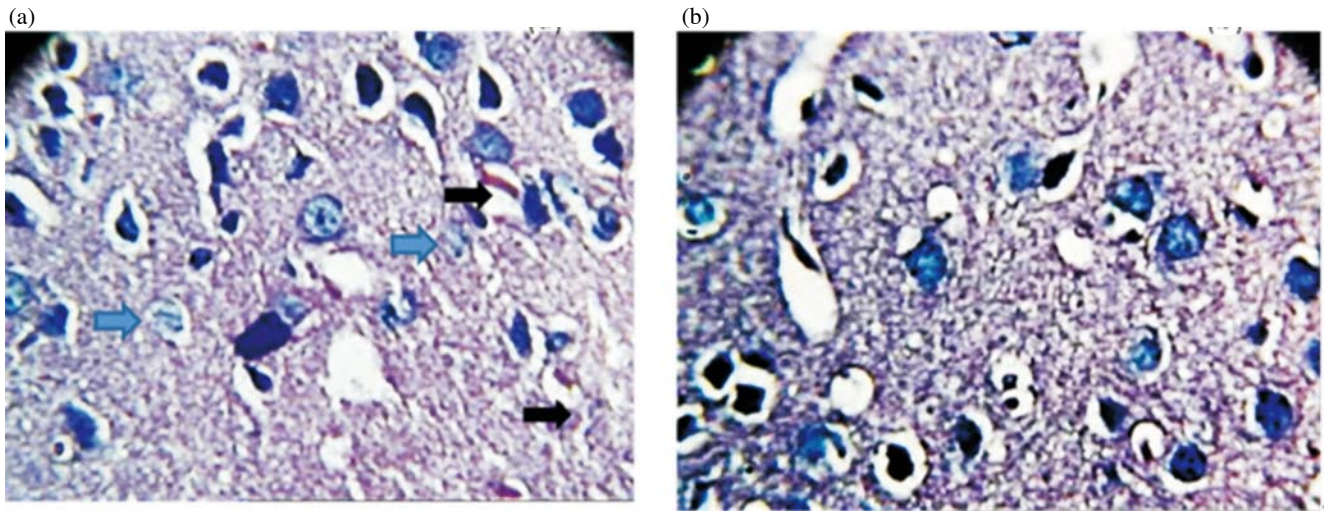
(a)   (b)

Figure 8. Brain tissues of Swiss albino mice untreated but infected with *Plasmodium berghei* ANKA (PbA) (a) and treated with nicotine (200 μg/mL) and (b) in drinking water daily for 6 weeks prior to PbA infection. Formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin (HE). The sections were photographed with a ×400 objective (bars are 15 μm). Black arrow shows sequestration of PRBCs in the cerebral micro vessels, while blue arrow shows necrosis of neurons.

nicotine (100 and 200 μg/mL) were alive, which didn't show any neurological syndromes including ataxia, paralysis, deviation of the head, convulsions, decrease in body temperature, and coma. Except mere presence of high parasitemia in their blood, high rectal temperature, gradual reduction in hemoglobin level and body weight, detectable deteriorating neurological symptoms were not observed in nicotine treated mice prior to PbA infection. Rather, they were active and running in their cage as usual while all control mice develop all neurological symptoms. Histological analysis of brain also indicated that lack of loss of vascular cell integrity, tissue edema, hemorrhages in the brain of nicotine treated mice, and congestion of micro-vessels with parasitized erythrocytes.

4. Discussion

Finding of this study showed that nicotine use in Swiss albino mice was positively associated with some life threatening malaria associated complications but not with cerebral malaria symptoms such as sudden reduction of body temperature, and neurological symptoms[20]. Also, histopathological changes involved in murine CM are characterized by loss of vascular cell integrity, tissue edema, bleedings in the brain of mice and blocking of small vessels with parasitized red blood cells and/or mononuclear cells were not observed[21,22]. Moreover, PbA infected Swiss albino mice develop neurological symptoms and cause 100% death[21].

However, lack of neurological symptoms associated to cerebral malaria in the Swiss albino mice treated with nicotine prior to PbA infection could be attributed to anti-inflammatory property of the nicotine. Although, there are many assumptions about pathogenesis of CM, the most accepted view is the elevated release of pro-inflammatory cytokines[23,24]. Excessive activation of pro-inflammatory cytokines by some parasite's components during malaria infection and *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) protein family are critical for pathogenesis of CM[25,26]. Even if, the exact cellular and molecular pathogenesis of CM is not known, the mechanical blockage of blood vessels by parasitized RBCs is widely accepted pathogenesis of CM. Since the adherence of parasitized erythrocytes to

endothelial cells ultimately leads to sequestration and obstruction of brain capillaries. The consequent reduction in blood flow causes cerebral hypoxia, localized inflammation and release of neurotoxic molecules and inflammatory cytokines by the endothelium[27].

When nicotine binds to its receptors (nRChRs), it inhibits inflammatory mediators[28,29]. Evidence showed that nicotine has anti-inflammatory property with ability to alter the capacity of cells to respond to the pro-inflammatory cytokine TNF-α or inhibited the release of this cytokine from the immune cell[29]. The anti-inflammatory activity of nicotine seems to occur through reduction of inflammation biomarkers, uric acid and CRP which triggers secretion of pro-inflammatory cytokines, mediators accountable for incidence of cerebral malaria pathology. Moreover, elevated level of such inflammation biomarkers were reported to have positive association with severe malaria pathologies in human and model animals.

In line to this, nicotine has been shown to reduce atopic disorders such as asthma and preeclampsia[30]. Mechanism of action in these cases may be, nicotine interfering with the inflammation-related disease process, as nicotine has vaso-constrictive effects[31]. Besides, nicotine might have effect on production of some immune cells mainly T-lymphocytes during the rodent malaria infection. This was more implicated by significant reduction of thymus weight in mice chronically treated with nicotine[32].

In agreement to the report of Rachid *et al.*[33], mice chronically treated with nicotine develop significant reduction in RBCs count, Hb and HCT levels. Decreasing level of such hematological parameters is a hallmark of anemia. Severe anemia is one of the life threatening malaria related health problems. Usually anemia is associated with intense hemolysis of infected RBCs due to higher parasitemia. However, in mice treated with nicotine prior to PbA infection, parasite load observed on Day 7 p.i. was lower than the positive control. According to Ingyang *et al.*[34], Swiss albino mice infected with PbA, develop parasitemia on the second day of p.i. and reach significant level from 4th day to 6th and all untreated mice died by the 6th day of infection. Thus, manifestation of symptoms of anemia could be attributed to the cytotoxic effect of nicotine than the parasite[35].

As monocytes and lymphocytes play a crucial role in the induction and maintenance of an immune response, ratio of the two WBC indices, monocyte-lymphocyte count ratio (MLCR), is lately considered as very key tool in prediction of an individual's protection against clinical manifestation of *Plasmodium falciparum* malaria[36]. Increased ratio value for MLCR is associated with severe malaria cases with reduced immunity[36]. MLCR analysis one in nicotine treated mice prior to PbA infection has showed significantly higher value of MLCR at higher concentration of nicotine than the positive control. This implies that, nicotine besides increasing susceptibility to severe malaria disease, it might be a risk factor for induction of protective malaria immunity. Since higher value of MLCR is associated to lower immunity against malaria vaccine under trial[37].

Thrombocytopenia, one of important pathologies associated with pathogenesis of severe malaria, is manifested by reduction of platelet count in peripheral blood. Its occurrence is when activated platelets during the course of malaria infection[38] release micro-particles that modulate the sequestration of parasitized erythrocytes (PRBC) on the brain endothelium, platelets aggregate[39-41]. Mechanism of platelets induced CM is, assumed that through contact dependent pro-apoptotic effect of platelets on TNF-α activated human brain micro-vascular endothelial cells (HBEC) [42]. The tumor growth factor (TGF)-β released from activated platelets induces the process of human brain endothelial cells killing[42]. Also platelets play great role in alteration of brain endothelium function[43]. Although, thrombocytopenia expected to appear as early as Day 4 PbA p.i.[44], in mice chronically treated with nicotine prior to PbA infection, insignificant incidence of thrombocytopenia was observed. Thus, as mediators of inflammations are suppressed by nicotine, activation of brain endothelial cells which attributes for sequestration of platelet and then leading to reduction of platelet count in peripheral blood in nicotine treated mice could be lower.

Pulmonary edema, one of the most severe forms of pathology of malaria that involve lung and characterized by increased alveolar capillary permeability could lead to intravascular fluid loss into the lungs[45]. Pulmonary edema is an indirect measurement of acute lung injury and acute respiratory distress syndromes. Like its parent compound, cigarette smoke[46], higher concentration of nicotine (200 μg/mL) was associated with higher incidence of pulmonary edema during PbA infection.

Nicotine is metabolized in liver by enzymer cytochrome P450, CYP2A6, and CYP2B6 into its major metabolite, cotinine and others[47]. Liver is also an important primary organ that involve in malaria infection, mainly during a hepatic stage of the parasite's life cycle, where malaria sporozoites develop into merozoites. Since in this study the parasite used for initiation of malaria infection was blood stage, destruction of liver by sporozoites can't be a cause for the liver biomarkers disorder. Rather when parasitized RBCs (PRBCs) loaded in the liver blood vessels or when the degraded haemozoin pigment during blood stage of malaria infection is engulfed by local tissue macrophages, such as Kupffer cells and alveolar macrophages, rupturing of these cells associated to conditions liver disorders such as jaundice, hepatomegaly and elevated liver enzymes[48,49]. But this condition can equally occur in all malaria infected mice (the positive control and nicotine treated mice). However, the significant elevated level of liver enzymes and reduction in level of albumin in mice treated with nicotine prior to PbA infection could be contributed to the damaging effect of nicotine as liver is a site for nicotine metabolism[48]. Thus, nicotine can increase relative risk to jaundice and hepatic impairment during malaria infection. This was more strengthened by necrosis of liver and kidney tissue observed in nicotine treated mice.

Finding of the study showed that chronic use of nicotine was associated with increases risk to some severe malaria symptoms, but not to neurological. Thus, like in respiratory infections, nicotine could increase susceptibility to some life threatening malaria syptoms. On the other hand, anti-inflammatory property of nicotine could be attributed to suppression of signs of CM.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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