Arrhythmogenic Risk Assessment Following Four-Week Pretreatment With Nicotine and Black Tea in Rat

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1. Background

Despite the known adverse effect of cigarette smoking on progression of atherosclerotic diseases, its role on cardiac arrhythmia is less clearly defined (1). Cigarette smoking may contribute to cardiovascular events by inducing a hypercoagulable state, reduction in the oxygen delivery capacity of the blood, endothelial dysfunction, and increase in catecholamine release and coronary vasoconstriction. Some of these mechanisms may contribute to the development of cardiac arrhythmia. Previous human studies suggest that the cardiac arrhythmia in smokers were not consequences of atherosclerosis at the coronary site, but rather a direct consequence of smoking (1-4).

Nicotine is one of the 4000 or more chemical components of tobacco smoke and many deleterious effects of cigarette smoke, such as cardiovascular diseases, are attributed to it (5). It is reported that chronic nicotine administration induces pro-oxidant/antioxidant imbalance (6). Some previous studies showed that chronic nicotine leads to damage of endothelium-dependent relaxation (7). Miyauchi et al. documented that nicotine increases heart propensity to arrhythmia by increasing the amount of interstitial collagen of atrial tissue in dogs with previous myocardial infarction (MI), but not in non-MI dogs. In this study, although the levels of nicotine in blood were similar in both groups and were within the range seen in smokers, interestingly, nicotine induced significant increase in atrial interstitial fibrosis in MI dogs, but only a mild increase in dogs with no MI (8). Another study showed that nicotine increases the amplitude and duration of action potential in the epicardial border zone of infarcted area of dog’s hearts, but not in the normal zone (9).

Moreover, other constituents of cigarette smoke such as carbon monoxide (CO) and oxidative stress may play a role in promoting cardiac arrhythmias. There is some evidence that confirmed the arrhythmogenic effect of
CO in human (10, 11) and animals (12). Recent studies suggest that oxidative stress increases the arrhythmogenic events (12, 13). On the other hand, numerous studies have demonstrated the positive effects of nicotine, including its neuroprotective effect in Parkinson and Alzheimer’s diseases (14, 15) and its therapeutic effect in ulcerative colitis (16). Even at low concentrations, nicotine enhances endothelial survival and release of endothelium-dependent vasodilators (17).

Mariscalco and Engstrom indicated that in comparison with nonsmokers, current smokers are less prone to develop atrial fibrillation after cardiac surgery (18). Despite all the above mentioned harmful and useful effects attributed to nicotine, today, nicotine replacement therapy is used as an effective intervention for smoking abstinence (19).

Black tea, as the most common beverages after water in the world (20), contains considerable amounts of flavonoid, which a part of its cardioprotective effect is attributed to this compound (21). Previous studies documented that in contrary to nonsmokers, cigarette smokers consumed more tea and coffee (22, 23). In a recent study, we demonstrated that black tea consumption may protect the cardiovascular system from some deleterious effects of nicotine including hyperlipidemia and atherogenesis, even without significant effects on heart antioxidant levels (24). In addition, based on hemodynamic, histopathologic, and biochemical indices, we showed that four-week administration of black tea or nicotine alone may attenuate isoproterenol-induced myocardial injury. Moreover, concomitant use of these materials did not show an additive cardioprotective effect (25).

2. Objectives

There is an uncertainty regarding the role of nicotine in arrhythmogenesis of cigarettes, the common concurrent consumption of black tea and nicotine-containing substances, and lack of adequate knowledge on the cardiac susceptibility to lethal ventricular arrhythmia following this consumption pattern. Therefore, we designed the present study to assess the validity of the proarrhythmic effect of nicotine and interaction of four-week coadministration of black tea and nicotine on cardiac vulnerability to life-threatening ventricular arrhythmia in an animal experimental model.

3. Materials and Methods

3.1. Animal Groups

This study protocol was approved by Ethic committee of Kerman University of Medical Sciences (permission No 90/475KA) and followed the national guidelines for conducting animal studies. This study included male Wistar rats aged three months and weighed 250 to 350 g, with careful implementation of animal care principles. Animals were weighed and randomized into control, tea, nicotine, and tea plus nicotine groups (N + T). The control group used tap water during experiment. Tea group used Lipton black tea brewed as their sole source of liquid for four weeks (25, 26). The method for preparation of black tea brewed was described previously (24, 25). In brief, it was prepared daily by soaking five Lipton teabags in 400 mL of boiling water for five minutes. Then the bags were removed, and the extract was allowed to cool for use. The average volume consumption of tea brewed by each rat was 32 ± 6 mL/d. The nicotine group received 2 mg/kg/d of nicotine subcutaneously and oral tap water for four weeks (24, 25, 27). The T + N group consumed tea along with nicotine with the same doses and methods used for tea and nicotine groups. In addition, normal saline (0.5 mL), as the vehicle of nicotine, was injected daily to control and T groups during the experiment.

3.2. Measured and Calculated Physiologic Parameters

On day 29, animals were weighed and anesthetized with intraperitoneal injection of sodium thiopental (50 mg/kg). The trachea was cannulated and animals were artificially ventilated with room air (at 50 strokes/min and stroke volume of 0.8 mL/100 g of body weight) during arrhythmia induction. The right common carotid artery was cannulated and connected to a pressure transducer and a PowerLab system (AD Instruments, Australia). The electrodes of electrocardiogram (ECG) lead II were attached to the limbs of the animals. The heart rate (HR), ECG, and arterial blood pressure (BP) were continuously recorded during the experiment under anesthesia. An angiocatheter (gage 24) was inserted into the lateral vein of the animal tail and then connected to a syringe containing arrhythmogenic drug (aconitine) by an appropriate tube. The time window for the animal recovery from surgery was 15 minutes and after that, the basal ECG and BP were recorded. The animals with cardiac arrhythmia or with a sustained drop in mean arterial pressure (MAP) < 70 mm Hg during the stabilization period were excluded from the study (28). The (MAP) was calculated as follows: MAP = diastolic BP + [(systolic BP - diastolic BP)/3]. Basal ECG parameters in each group were measured by mean of one-minute recorded strip. Corrected QT (QTc) interval was calculated using Bazett’s formula normalized as QTcn-B = QT/ (RR/f)0.5, where RR is R-R interval and f = 150 ms (28, 29).

3.3. Arrhythmia Induction

After the time window and baseline recording, aconitine solution was infused for arrhythmia induction via the tail vein with a syringe pump at a velocity of 0.1 mL/min (15 μg/mL in saline) for ten minutes (28, 30). The BP and ECG were simultaneously recorded from the onset of infusion through five minutes after the end of infusion (totally, 15 minutes). During the 15 minutes of the test, the episodes of total premature ventricular contractions...
(PVCs) (PVC + Salvo) and ventricular tachycardia (VT) plus ventricular fibrillation (VF) were counted and the latency and duration of VT + VF in seconds were measured.

According to the Lambeth conventions, ventricular arrhythmias were defined as follows:

Ventricular premature beat (VPB) or PVC: a ventricular electrical complex (complete electrical event, QRS, RSQST, or RST) that is different in shape (voltage and/or duration, ie, height and/or width) from the preceding (non-VPB) ventricular complex, and is premature in relation to the preceding ventricular complex.

Salvo: two or three consecutive VPBs. VT, a run of four or more consecutive ventricular premature beats. VF, a sequence of a minimum of four consecutive ventricular complexes without intervening diastolic pauses in which the intrinsic shape, the peak–peak interval and the height vary, and the variation between each is non-progressive (31).

The threshold dose of aconitine required for producing lethal ventricular arrhythmias (VT + VF) were determined according to the following formula: Threshold dose (μg/kg) for arrhythmia = 15 μg/mL × 0.1 mL/min × time required for arrhythmia (min)/body weight (kg) = 15 μg/min × time (min)/body weight (kg). Threshold dose of 100 μg/kg and latency time of 1000 seconds were considered for animals without VF. In addition, the severity of arrhythmias in the different groups was presented quantitatively by a previous scoring system defined as follows:

0, < 10 PVCs; 1, > 10 PVCs; 2, one to five episodes of VT; 3, > 5 episodes of VT or one episode of VF; 4, two to five episodes of VF; 5, > 5 episodes of VF; 6, VT, VF, or both with duration > 300 seconds (32). Sodium thiopental was purchased from Sandoz, Austria, Nicotine and Aconitine from Sigma, the United Kingdom, and black tea from Lipton, Inc, the United States.

3.4. Redox Indices

Animals were killed immediately after arrhythmia recording and the hearts were removed and washed with cold saline. A piece of the heart apex was dissected, weighted, and homogenized in 5 mL of 0.1 M Tris–HCl buffer (pH 7.4) in ice-cold condition. The samples were centrifuged and the supernatants were separated for the biochemical analysis. The amounts of total proteins were measured by using the Lowry et al. method (33). Malondialdehyde (MDA) level, as an index of lipid peroxidation, was estimated by concentration of thiobarbituric acid reactive substances (TBARS) (34). Total antioxidant capacity (TAC) was determined by using their relative Randox assay kits according to the manufacturer’s protocol (35). The TAC to MDA ratio, as an index of oxidative stress status, was measured in all groups (36).

3.5. Statistical Analysis

Regarding the exclusion criteria, only data from surviving rats were analyzed. At the end of experiment, seven to eight animals in each group survived that was adequate for animal studies based on similar previous researches and ethics in animal experiments. The results were presented as mean ± SD and the normal distribution of data was confirmed by Kolmogorov-Smirnov test. Comparison of biochemical parameters, HR, BP, arrhythmia episodes, arrhythmia duration, latency, and threshold dose of aconitine among different groups was performed using one-way ANOVA and post hoc Tukey’s test. Scores of arrhythmia severity in the animal groups were compared using non-parametric Kruskal Wallis and Mann-Whitney U tests. P value < 0.05 was considered as statistically significant. The data was analyzed by use of SPSS 20 (SPSS Inc., Chicago, Illinois, the United States).

4. Results

4.1. Weight Gain, Blood Pressure, and Heart Rate

Administration of tea and nicotine, alone or in combination, had significant inverse association with body weight gain. At the end of the study, the control group had 17% weight gain, while tea and nicotine groups showed respectively 6.7% and 10% weight gain, and N + T showed 10% weight reduction. Weight differences between the control group and other groups were statistically significant (P < 0.001). In addition, nicotine alone or in combination with tea showed more negative effect on weight gain (Table 1). Overall, BP was higher in the nicotine group and was lower in the tea group, but the differences were not statistically significant. The HR showed none significant decrease in all groups compared to control group (Table 1).

4.2. Electrocardiogram Parameters

The RR, PR, and QRS intervals did not show significant difference among animal groups. However, pretreatment with tea and nicotine, alone or in combination, was associated with degrees of QTcn and JT prolongation (Table 1 and Figure 1). The QTcn significantly increased in nicotine group (P < 0.01 and P < 0.05 versus control and Tea group, respectively) and N + T group (P < 0.01 compared with control and tea groups). Prolongation of the JT interval was significant in all groups in comparison with control group (P < 0.05 for tea, and P < 0.01 for nicotine and N + T groups). As shown in Table 1 and Figure 1, combination of tea and nicotine showed additive incremental effects on these parameters.

4.3. Susceptibility to Ventricular Arrhythmias

Four-week consumption of black tea resulted in significant increase in the number of PVCs when compared with the control group (P < 0.01) (Figure 2 A). Although tea and nicotine alone or in combination had no significant effect on the numbers of VT + VF or VF (Figure 2, B), but tea and nicotine individually decreased the VT + VF duration (P < 0.001 for tea group and P < 0.05 for nicotine group compared with control group) (Figure 2 C).
Table 1. Weight Changes, Blood Pressure, Heart Rate, RR, PR, QRS, and JT Intervals in Electrocardiogram of the Animal Groups a,b

| Group   | n | WT Changes, % | SBP, mm Hg | DBP, mm Hg | MAP, mm Hg | HR, beat/min | RR, ms | PR, ms | QRS, ms | JT, ms |
|---------|---|---------------|------------|------------|------------|--------------|--------|--------|---------|--------|
| CTL     | 7 | 17 ± 2.4      | 120 ± 13.1 | 100 ± 10.9 | 106 ± 11   | 412 ± 21.4   | 146 ± 7.5 | 44 ± 3.9 | 19 ± 3.6 | 33 ± 8.4 |
| Tea     | 8 | 6.7 ± 3.9 c   | 118 ± 17.7 | 97 ± 18.2  | 104 ± 17.8 | 376 ± 36.1   | 161 ± 16.1 | 44 ± 2.39 | 17.1 ± 1.83 | 39.4 ± 6.9 d |
| N       | 7 | 1 ± 4.3 e,f   | 140 ± 17.5 | 115 ± 14.3 | 124 ± 14.8 | 381 ± 26.4   | 157 ± 10.63 | 44.7 ± 3.44 | 18.4 ± 2.27 | 45.4 ± 3.3 g |
| T + N   | 7 | -10 ± 4.2 h   | 135 ± 28.4 | 108 ± 24.2 | 117 ± 25.5 | 386 ± 29.5   | 155 ± 12.7 | 44.2 ± 3.6  | 17.9 ± 1.7 | 47.4 ± 6.8 g |

P value 0.000 0.074 0.144 0.109 0.153 0.171 0.960 0.605 0.001

a Abbreviations: CTL, control group; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; N, nicotine group; n, frequency of animals in each group; SBP, systolic blood pressure; T, black tea group; T + N, black tea plus nicotine group; WT, weight.

b P < 0.001 versus control group.

c P < 0.001 versus control group.

d P < 0.05 versus control group.

e P < 0.001 versus control group.

f P < 0.05 versus Tea group.

g P < 0.001 versus control group.

h P < 0.001 versus other groups.

The latency times from the onset of aconitine infusion to the first VT and VF were measured in the different groups. The latency for first VT event was significantly longer in the tea and nicotine groups (P < 0.001) as well as N + T group (P < 0.01) than in control group. This pattern was repeated for VF latency, but it was only significant in tea group (P < 0.01) and nicotine group (P < 0.05) versus control group (Figure 3 A). In addition, threshold dose of aconitine for induction of VT and VF was enhanced in all test groups, but it was significant only for VT (P < 0.001 whenever all groups were compared with the control group) (Figure 3 B). The score of arrhythmia severity was diminished in tea group (P < 0.001) and nicotine group (P < 0.01) compared to the control group. However, in presence of combination of tea and nicotine, the scores of lethal arrhythmia showed no significant difference in comparison with control group (Figure 2 D).

4.4. Redox Findings

Black tea, nicotine, and their combination increased the TAC level of heart, but this parameter was only significant in tea group whenever compared with control group (P < 0.01). The MDA levels also increased in all test groups (tea, nicotine and T + N groups); however, it was significant in nicotine and T + N groups in comparison with control group (P < 0.01). The TAC to MDA ratio was significantly higher in animals’ hearts that received black tea than in both control and T + N groups (P < 0.05) (Table 2).

5. Discussion

Although the major component of tobacco smoke involved in the pathogenesis of cardiac arrhythmia is still unclear, nicotine is introduced as the main responsible component for arrhythmogenesis of tobacco in some studies. The present study was conducted to determine the validity of the proarrhythmic effect of nicotine and the outcomes and interaction of intermediate-term, four-week coadministration of black tea and nicotine on susceptibility to life-threatening ventricular arrhythmia in rats.

The results showed that four-week administration of nicotine and black tea, alone or in combination, significantly prevents the animals’ weight gain. Moreover, these agents alone or in combination had no significant effects on BP and HR, but increased the QTC and JT intervals. In addition, black tea and nicotine individually reduced the score of arrhythmia severity. Combination of these agents had no significant effect on number, duration, and severity score of dangerous arrhythmias.

In agreement with our findings, the negative effect of black tea or nicotine on the animals’ weight gain is reported in previous studies. In a study, chronic black tea extract (50 g in 800 mL) consumption decreased body weight gain of rats by 17.8% (37). In another study, we observed similar results (25). Recent reports suggest that black tea may prevent diet-induced hyperlipidemia and obesity through inhibiting intestinal lipid absorption (38). Negative effect of nicotine on body weight gain is also indicated in several studies (39, 40). Dandekar et al. suggested that nicotine-induced weight gain limitation is the result of reduced food intake rather than increased metabolism (41).

The present finding about animals’ BP is consistent with some human studies and our previous experimental study that demonstrated four-week consumption of black tea and nicotine alone or simultaneously had no significant effects on BP (25). The results of a single blind, crossover, placebo-controlled study indicated that high-dose transdermal nicotine along with cigarette smoking had no additional adverse effects on HR, BP, fibrinogen levels, or lipid profiles in long-term smokers (42). Moreover, findings of a study on a group of smokers revealed that oral and/or transdermal nicotine did not affect the BP or hematologic and coagulation indices (43). However, others reported that high, but no low or moderate, doses of nicotine increase catecholamine release (39) and hence, increase HR, BP, and susceptibility to cardiovascular events (44).
To the best of our knowledge, no study in the literature has directly investigated the effects of tea and nicotine on ECG parameters and studies in this field are incomplete. There is some evidence that chronic nicotine exposure may decrease $\text{Na}^+$/K$^+$ ATPase electrogenic activity and elevate resting membrane potentials. Elevated resting membrane potentials decrease inward Na$^+$ current (45). Given that aconitine-induced arrhythmogenesis results from Na$^+$ channel hyperactivation, reduction of inward Na$^+$ current may explain the nicotine’s antiarrhythmic effect, which was observed in the present study. Results of Wang et al. study revealed that extracellular applications of nicotine blocked the inward potassium channel (I_K1) of canine ventricular myocytes by direct interactions with the channels with a concentration-dependent manner. Moreover, they demonstrated that this effect occurs in the absence of nAChRs stimulation and catecholamine release, probably through the direct interactions between nicotine and potassium channels (46). Block of Kir channels, particularly the outward I_K1 currents, can explain the QTcn and JT interval prolongation in nicotine groups of our study. The depression of I_K1 channels can delay the late phase of membrane repolarization and hence, increased QT and JT intervals. This effect of nicotine is similar to the actions of Class III antiarrhythmic drugs (47), and explains the anti-
arrhythmic effect of this agent that was observed in the present study. However, bearing in mind that very long QT interval may cause early after depolarizations (EADs) due to activation of inward depolarizing currents. In turn, it can cause ventricular extrasystoles and finally, increase the

![Figure 1. Basal QT Interval and QT as Bazett’s Formula Normalized (QTcn) in Each Animal Groups](image1)

Data are presented as mean ± SD (n = 7 to 8 for each group); *, P < 0.05 versus tea group; †, P < 0.01 versus control group; ‡; P < 0.001 versus control group; •, P < 0.01 versus tea group; #, P < 0.01 versus control and tea groups. Control, control group; T, black tea group; N, nicotine group; N + T, nicotine + black tea group.

![Figure 2. The Effects of Four-Week Pretreatment of Nicotine and Black Tea on Number (A and B), Duration (C) and Score (D) of Ventricular Arrhythmia in Animal Groups](image2)

Data are presented as mean ± SD, (n, 7 to 8 for each group); •, P < 0.001 versus CTL group, ᶦ, P < 0.05 versus control group; *, P < 0.05 versus tea group; **, P < 0.01 versus CTL group;CTL, control group; T, black tea group; N, nicotine group; N + T, nicotine + black tea group.
risk of re-entry and polymorphic ventricular tachycardia, torsade de points (48, 49), an adverse effect of some drugs such as amiodarone from Class III antiarrhythmic drugs (50, 51). There is evidence that reactive oxygen species (ROS) scavengers are associated with successful decrease in the reperfusion-induced arrhythmias (52). Although the level of MDA significantly increased in the nicotine group, but the TAC to MDA ratio, as a new marker of oxidative stress, showed an insignificant incremental trend in comparison to the control group. This means that in the present study, nicotine consumption was not associated with the oxidant/antioxidant balance perturbation in rats’ heart. In an experimental study, intravenous injection of nicotine to anesthetized dogs with low doses (2.5, 5, and 10 µg/kg) did not induce significant arrhythmia; however, higher doses of nicotine (> 50 µg/kg) was associated with supraventricular and ventricular arrhythmias (53). In the present study, we administered 2 mg/kg/d of nicotine subcutaneously for four weeks. Therefore, the disparity in the animal species, the route and duration of nicotine administration, and the model of arrhythmia induction are probable reasons for the differences between these studies.

A part of antiarrhythmic effect of black tea may come from its QT-interval prolongation effect and hence, delay in repolarization period of cardiac myocytes, which were observed in our study. On the other hand, black tea significantly increased the TAC level and TAC to MDA ratio of rats’ heart. Regarding the proarrrhythmic effect of oxidative stress (12, 13) and antiarrhythmic effect of antioxidants, a part of the antiarrhythmic effect of black tea may be induced by its antioxidant properties and stabilizing the redox balances. In addition, Short-term and long-term black tea consumption reverses endothelial vasomotor dysfunction in patients with coronary artery disease. This effect may partly explicate the association between tea intakes and decreased cardiovascular events (54). However, due to the lack of adequate information, further studies are needed to understand the exact mechanism of this finding. Increase in the number of PVCs in tea group may results from the effect of caffeine in tea that can induce benign arrhythmias in small dose (55, 56). Obviously, the mere increase in PVC, without re-entry or trigger activity phenomena, cannot lead to fatal cardiac arrhythmias.

**Table 2.** Total Antioxidant Capacity, Malondialdehyde, and Total Antioxidant Capacity to Malondialdehyde Ratio of Heart Tissue in Different Groups a,b

| Group | n   | TAC, µmol/mg protein | MDA, nmol/mg protein | TAC/MDA |
|-------|-----|---------------------|---------------------|---------|
| CTL   | 7   | 0.029 ± 0.002       | 0.122 ± 0.026       | 247 ± 56 |
| T     | 8   | 0.083 ± 0.04 c      | 0.156 ± 0.037       | 590 ± 353 d |
| N     | 7   | 0.057 ± 0.029       | 0.194 ± 0.048 e     | 322 ± 176 |
| T + N | 7   | 0.050 ± 0.014 f     | 0.193 ± 0.025 f     | 246 ± 66 g |

Data are presented as mean ± SD, (n, 7 to 8 for each group); •, P < 0.001 versus control group; *, P < 0.05 versus control group; **, P < 0.01 versus control group; Control, control group; T, black tea group; N, nicotine group; T + N, black tea plus nicotine group.

Abbreviations: TAC, total antioxidant capacity; MDA, malondialdehyde; CTL, control group; T, black tea group; N, nicotine group; T + N, black tea plus nicotine group.

A part of antiarrhythmic effect of black tea may come from its QT-interval prolongation effect and hence, delay in repolarization period of cardiac myocytes, which were observed in our study. On the other hand, black tea significantly increased the TAC level and TAC to MDA ratio of rats’ heart. Regarding the proarrrhythmic effect of oxidative stress (12, 13) and antiarrhythmic effect of antioxidants, a part of the antiarrhythmic effect of black tea may be induced by its antioxidant properties and stabilizing the redox balances. In addition, Short-term and long-term black tea consumption reverses endothelial vasomotor dysfunction in patients with coronary artery disease. This effect may partly explicate the association between tea intakes and decreased cardiovascular events (54). However, due to the lack of adequate information, further studies are needed to understand the exact mechanism of this finding. Increase in the number of PVCs in tea group may results from the effect of caffeine in tea that can induce benign arrhythmias in small dose (55, 56). Obviously, the mere increase in PVC, without re-entry or trigger activity phenomena, cannot lead to fatal cardiac arrhythmias.

**Figure 3.** The Effects of Four-Week Pretreatment of Nicotine and Black Tea on Latency, Times From the Onset of Aconitine Infusion to the first VT and VF (A), and Threshold Dose of Aconitine for Induction of VT and VF (B)

![Figure 3](image-url)
As mentioned above, combination of tea and nicotine eliminated its antiarrhythmic effects. Much longer QT interval can be considered as a possible reason for the abolition of antiarrhythmic properties in N + T group. As previously described, very long QT interval via activation of inward depolarizing currents, may cause EADs, which in turn can trigger ventricularextrasystoles and increases the risk of re-entry and polymorphic VTs (48, 49). In addition, the collective stimulating effect of these agents on sympathetic system and increasing the catecholamine release (57, 58) may mask its antiarrhythmic effects that was observed in this study.

Overall, the findings suggest that four-week administration of black tea or nicotine alone, with dosages used in this study, may decrease the susceptibility to life-threatening ventricular arrhythmia. However, concurrent use of these agents had no significant effects on propensity to malignant arrhythmias. Therefore, intermediate-term consumption of either nicotine or black tea with appropriate doses has potential antiarrhythmic effects and their combination regimen does not increase the risk of fatal ventricular arrhythmias in rats. Extrapolation of these findings to humans and consequences of long-term consumption of tea and nicotine on susceptibility to cardiac arrhythmias requires further studies.

Authors’ Contributions
Elham Ghasemipoor Afshar and Soodabe Ghorbani Shahrbabaki contributed to the preparation and treatment of animals and performing experimental procedure and sampling. Siyavash Joukar developed the original idea and the protocol, supervised the study, performed experimental procedures and data analysis, and wrote the manuscript. Vahid Sheibani and Faramarz Koushesh were design consultants.

Funding/Support
Kerman University of Medical Sciences and Health Services (KUMS), Kerman, Iran, provided financial support for this study.

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