Influence of betel quid on effect of calcium channel blockers on isoprenaline
induced myocardial necrosis in mice

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It is known that chewing Betel quid with tobacco (BQT) or without tobacco (BQ) is a major etiological factor for cardiovascular complications and calcium channel blockers (CCBs) are the major class of drugs prescribed widely for myocardial disturbances. The possible pharmacodynamic interaction between CCBs (verapamil, amlodipine and diltiazem) and BQ/BQT was studied on isoproterenol (ISO)-induced myocardial necrosis in mice. Influence of (CCBs) therapy on pretreated animals at times of myocardial stress were determined by estimating diagnostic marker enzymes such as lactate dehydrogenase (LDH) and creatine phosphokinase isoenzyme (CK-MB) in serum and heart tissue homogenate (HTH). Administration of CCBs to mice pretreated with BQ produced a significant decrease and increase in biomarker enzyme levels in serum and HTH respectively. Further, incorporation of diltiazem and amlodipine in BQT pretreated mice significantly elevated enzyme levels in HTH, whereas, amlodipine administration during BQT treatment showed significant fall in enzyme levels in serum. The results indicated that BQT is cardiotoxic and its effect cannot be reversed using CCBs while BQ is cardioprotective, whose activity was further augmented by amlodipine. Histopathological studies confirmed the biochemical findings.

Keywords: Amlodipine, Betel quid, Calcium channel blockers, Diltiazem, Tobacco, Verapamil

The habit of chewing betel quid containing fresh, dried or cured areca nut, catechu, slaked lime and flavoring ingredients wrapped in betel leaf is widespread in India, Pakistan, Bangladesh and Sri Lanka and in migrant populations from these regions in other countries. Betel quid chewing is known to produce many pathological changes in the body. It is one of the leading cause for development of oral cancer and oropharyngeal tumors. It can also lead to cholinergic crisis, cardiac arrhythmias, mild psychosis, milk-alkali syndrome, neurological disorders and gastrointestinal problems to name few of its effects. Besides these, the tobacco and slaked lime present in betel quid can increase plasma concentrations of norepinephrine; epinephrine and can induce hypercalcemia and metabolic alkalosis. Further, arecanut, a component of betel quid is shown to induce significant increase in the levels of cytochrome b5, cytochrome P-450, glutathione s-transferase and malondialdehyde (MDA) in dams and their pups. Areca is also reported to increase activity of endogenous antioxidant enzymes. On the contrary, tobacco, which is also a constituent of betel quid in many parts of the world, is reported to induce production of oxygen free radicals.

The whole betel quid chewing is known to induce myocardial necrosis, cardiac arrhythmias and sympathetic activation. Since, calcium channel blockers are one of the classes of drugs used in the treatment of myocardial ischemia, cardiac arrhythmias and hypertension, the present study has been undertaken to investigate the interaction between calcium channel blockers and betel quid without tobacco (BQ) or betel quid with tobacco (BQT) in isoprenaline (ISO) induced animal model of myocardial necrosis.

Materials and Methods

Experimental animals— Male Swiss albino mice weighing between 25-30 g were used. All animals received humane care and Institutional Animal Ethics Committee approved the experimental protocol (Reference number -KCP/IAEC-01/2006, date of issue: 07/03/2006). Animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).
Chemicals and reagents—All chemicals used were of analytical grade and purchased from standard companies. Biochemical kits like lactate dehydrogenase (LDH) and creatinine kinase-MB (CK-MB) were procured from Crest Biosystems (Goa, India).

Preparation of betel quid extract and selection of dose—Preparation of betel quid (without tobacco) extract: The Kolkata pan (betel leaves), a variety of Piper betel, were purchased from the local market. It was identified and authenticated by Mrs. Shalini Kapoor Mehta, Department of Pharmacognosy, Krupanidhi College of Pharmacy, Bangalore. Around 500 betel leaves (weighing 1.45 kg) were cut into small pieces and grinded with 3.5 liters of distilled water to make a paste like mass. Slice cut areca nuts (500 g) was powdered. Finally the betel leaf paste, powdered areca nut and 60 g of slaked lime were mixed well to make a paste and this was kept for 6 h at -20°C. The mixture was then filtered and the filtrate was freeze-dried and stored at -20°C till use. The proportions of the constituents were selected after consulting local betel sellers.

Preparation of betel quid (with tobacco) extract: Around 500 betel leaves (weighing 1.45 kg) were cut into small pieces and grinded with 3.5 liters distilled water to make a paste like mass. Slice cut areca nuts (500 g) and 312.5 g tobacco (mash mash brand khamin) was powdered. Finally the betel leaf paste, powdered areca nut, tobacco and 60 g of slaked lime were mixed well to make a paste. The paste was stored for 6 h at -20°C C. The mixture was then filtered and the filtrate was freeze-dried and stored at -20°C till use.

Selection of doses and treatment period: The yield of freeze dried extract was found to be 208 g. The extract was reconstituted with distilled water and a single dose of 13 mg/kg po was administered to mice for one month. The doses of BQ or BQT were selected on the basis of a consumption of betel quid by human per day, which is around 5-7 leaves per day as per the market survey. The dose for mice was selected from the yield of extract. The human dose was converted to mouse dose using the table described by Ghosh. The oral doses of calcium channel blockers were selected based on earlier reports (amlodipine-2.5 mg/kg/day, diltiazem hydrochloride-30 mg/kg/day and verapamil hydrochloride-30 mg/kg/day).

Experimental protocol—The animals were divided into following treatment groups consisting of at least nine animals in each group at the end of the treatment period. The first group (I) served as control group. The animals of group II and III received BQ and BQT orally at a dose of 13 mg/kg for 5 weeks. The groups IV, V and VI animals were treated orally for 7 days with three different classes of calcium channel blockers because each of the drugs has different pharmacological actions. The mice of group IV received verapamil, a potent cardiac depressant with very little vasodilation. The group V was given diltiazem, moderate cardiac depressant. The group VI was administered amlodipine, a dihydropyridine with good vasodilator effect and little or no cardiac depressant actions. The animals of group VII, VIII and IX were administered BQ for 5 weeks and during last 7 days of BQ treatment, calcium channel blockers - verapamil, diltiazem or amlodipine was administered orally.

At the end of treatment period, animals of all the groups were administered isoprenaline (ISO) initially at a dose of 250 mg/kg sc followed by 2 consecutive doses of 125 mg/kg sc for the next 2 days. The dose for induction of myocardial necrosis was determined in our lab and this dosing of ISO produces significant myocardial necrosis when determined by both biochemical and histopathological examinations. Symptoms of mortality in each group were recorded and compared with those of mice given ISO alone. After 72 h of the first ISO administration, blood was withdrawn and serum was separated. The activities of lactate dehydrogenase (LDH) and creatine phosphokinase isoenzyme (CK-MB) were determined in the serum. The mice were sacrificed and autopsied. The hearts were removed and weighed; five of the nine hearts were taken for the homogenate preparation and remaining four were taken for histopathological examination to determine necrosis.

For histopathological studies, hearts were immersed in 8% formalin solution in saline. The tissues were then dehydrated using graded doses of ethanol starting from 70% ethanol to absolute ethanol, followed by clearing and infiltration. The whole hearts were then embedded in paraffin wax and 4-µm-thick cross transverse sections was made at the basal, mid, and apical levels of the complete heart. These sections were stained with hematoxylin and eosin (H & E). The extent of damage was calculated by grading the H & E stained mice heart sections as follows.
Five-point scale (0–4); one point each was scored for increased interstitial space, increased myocyte size, nuclear changes (hyperchromasia and/or duplication) and infiltration of leucocytes.

Also, the volume fraction of interstitial space (VFITS) in myocardial tissue was determined from H & E stained transverse sections by using the equation:

\[
\text{VFITS} = \frac{(100\% \times \text{area of interstitial space})}{\text{total tissue area}}
\]

The heart tissue homogenate was prepared in ice-cold 0.25\(M\) sucrose (1:10, w/v) for estimation of CK-MB and LDH levels using commercial kits.

**Statistical analysis**— The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. For comparing the myocardial necrosis scores, ANOVA followed by Non-parametric Dunn post test was used. The values were expressed as mean±SE and \(P<0.05\) was considered significant.

**Results**

**Effect on serum LDH and CK-MB**— The results are given in Table 1. There was significant (\(P<0.001\)) decrease in LDH levels in groups treated with BQ (group II), diltiazem (group V), amlodipine (group VI), BQ+verapamil (group VII), BQ+diltiazem (group VIII), BQ+amlodipine (group IX) and BQT+amlodipine (group XII) when compared to control. On comparing the LDH levels of CCB alone with BQ+CCB and BQT+CCB treated groups it was found that, BQ+CCB treated groups showed a significant (\(P<0.001\)) decrease in LDH levels and on the contrary BQT+CCB showed significant (\(P<0.001\)) increase in LDH level. The CK-MB levels in the serum were significantly (\(P<0.001\)) decreased in groups VI to IX when compared to control. The CK-MB level was significantly (\(P<0.001\)) reduced in groups VII to IX compared to group IV to VI respectively. Comparison of CK-MB levels of CCB alone with BQ + CCB treated animals and BQT+CCB treated animals showed that there was a significant decrease in the CK-MB levels in all the BQ+CCB treated groups compared to CCB treated groups.

**Effect on LDH and CK-MB in the heart homogenate**— The results are given in Table 1. A significant increase in the CK-MB levels was found in all the treatment groups except groups III, IV and X when compared to control. On comparing the CK-MB levels of CCB alone with the BQ+CCB and BQT+CCB treated groups; there was no significant alteration in the enzyme levels in any of the groups. Also, a significant increase in the LDH levels was found in all the treatment groups except group III. Comparison of LDH levels of CCB treated groups with the BQ+CCB and BQT+CCB treated groups revealed no significant alteration in the enzyme levels in any of the groups.

**Effect on VFITS (%)**—The results are given in Table 2. A significant increase in VFITS (%) was

<table>
<thead>
<tr>
<th>Group no</th>
<th>Treatment</th>
<th>LDH (U/l)</th>
<th>CK-MB</th>
<th>Heart homogenate (U/mg of the tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>2661.4±53.56</td>
<td>667.12±34.46</td>
<td>76.82±1.94</td>
</tr>
<tr>
<td>II</td>
<td>BQ</td>
<td>1718.4±37.62***</td>
<td>609.12±7.46</td>
<td>266.14±5.36***</td>
</tr>
<tr>
<td>III</td>
<td>BQT</td>
<td>2814.4±54.50</td>
<td>645.04±33.14</td>
<td>127.26±22.84</td>
</tr>
<tr>
<td>IV</td>
<td>Verapamil</td>
<td>2337.2±92.63</td>
<td>653.36±43.16</td>
<td>156.96±3.67</td>
</tr>
<tr>
<td>V</td>
<td>Diltiazem</td>
<td>1552.4±165.77***</td>
<td>602.84±19.65</td>
<td>172.65±26.80</td>
</tr>
<tr>
<td>VI</td>
<td>Amlodipine</td>
<td>1233.8±158.7***</td>
<td>561.16±10.34</td>
<td>247.5±5.96***</td>
</tr>
<tr>
<td>VII</td>
<td>BQ + verapamil</td>
<td>842.67±128.04***</td>
<td>373.42±18.66***</td>
<td>224.86±8.36***</td>
</tr>
<tr>
<td>VIII</td>
<td>BQ + diltiazem</td>
<td>648.63±166.43***</td>
<td>280.46±16.32***</td>
<td>243.4±5.60***</td>
</tr>
<tr>
<td>IX</td>
<td>BQ + amlodipine</td>
<td>635.98±118.74***</td>
<td>234.24±25.35***</td>
<td>280.35±0.37***</td>
</tr>
<tr>
<td>X</td>
<td>BQT + verapamil</td>
<td>2654.4±117.34***</td>
<td>713.00±18.04</td>
<td>172.64±27.0***</td>
</tr>
<tr>
<td>XI</td>
<td>BQT + diltiazem</td>
<td>2255.4±54.17***</td>
<td>661.96±14.04</td>
<td>268.46±12.8***</td>
</tr>
<tr>
<td>XII</td>
<td>BQT + amlodipine</td>
<td>1800.0±154.33***</td>
<td>631.42±11.42</td>
<td>228.3±16.22***</td>
</tr>
</tbody>
</table>

\(P\) values: *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\)

*: compared to control group; \(a, b, c\): compared to corresponding calcium channel blocker alone

BQ = Betal quid; BQT = Betal quid with tobacco
observed in the control group when compared to normal. There was a significant decrease in the VFITS (%) in all the treated groups except the BQT+verapamil treated group. On the contrary a significant increase in the VFITS (%) was observed in BQT treated group when compared to control. On comparing the VFITS (%) of CCB alone with the BQ+CCB and BQT+CCB treated groups, a significant decrease in the VFITS (%) was found in the BQ + amlodipine treated group.

Effect on extent of myocardial injury (EMI)—The results are given in Table 2. The extent of myocardial injury (EMI), in terms of grading (0-4), revealed a significant injury to the myocardium in the control group mice when compared to normal mice. The administration of ISO found to cause massive infiltration of leucocytes with nuclear duplication and hypertrophied myocyte (Fig. 2). No significant interactions could be seen in EMI between CCB treated group and BQ+CCB and BQT+CCB treated groups (Table 2). There was variable degree of infiltration of leucocytes with nuclear duplication and enhanced myocyte size in all pretreated groups except group IV and XI (Fig. 3). A representative figure (Fig. 4) of BQ + verapamil is given which shows mild degree of necrosis with infiltration of leucocytes.

Effect on mortality and body weight—The extent of mortality after ISO administration in BQT and BQT+verapamil treated groups was higher when compared to other treatment groups (Table 2). Mice receiving BQT had shown reduction in their body weight, and the muscular mass was also reduced. The mice treated with amlodipine and diltiazem during their 5th week, were successful in regaining their body weight, whereas verapamil treatment during the 5th week did not reverse the decreased body weight (data not shown).

Discussion
Chewing BQ with or without tobacco continues to be a major etiological factor for cardiovascular complications such as myocardial necrosis, hypertension and arrhythmias in various parts of the world. CCBs are the major class of drugs that are widely prescribed for most of these indications. Hence it is widely accepted that in-depth and appropriate studies on BQ/BQT with CCBs (diltiazem; verapamil and amlodipine) interactions should be carried out to confirm the efficacy/toxicity of combined usage.

BQ chewing is a major problem in south Asian population. Despite several reports on the hazardous effect of BQ and BQT chewing, it is widespread in different parts of India. Although BQ is shown to induce hypertension and myocardial damage, patients suffering from cardiovascular complications continue to consume BQ either with or without tobacco. One of the constituent of BQ and BQT, slaked lime [Ca(OH)₂] is reported to induce hypercalcemia. Apart from this, other constituents of BQ namely betel leaves and areca were also suspected to contribute to the hazardous effect of BQ. The BQT contains chewable tobacco in addition to constituents of BQ, and tobacco is one of the known causes for development of myocardial damage. Hence it has become a confirmed fact that people chewing BQ or BQT are susceptible to develop cardiovascular complications.

Table 2—Effect on volume fraction of interstitial space (VFITS), extent of myocardial injury (grading) and mortality.

<table>
<thead>
<tr>
<th>Group no</th>
<th>Treatment</th>
<th>VFITS (%)</th>
<th>Grading</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>2.58±0.3</td>
<td>0.33±0.21</td>
<td>---</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>12.00±0.7</td>
<td>3.16±0.16</td>
<td>1/10</td>
</tr>
<tr>
<td>III</td>
<td>BQ</td>
<td>3.5±0.1***</td>
<td>0.98±0.21</td>
<td>0/9</td>
</tr>
<tr>
<td>IV</td>
<td>BQT</td>
<td>15.0±1.2†</td>
<td>3.83±0.16</td>
<td>2/12</td>
</tr>
<tr>
<td>V</td>
<td>Verapamil</td>
<td>7.6±0.7</td>
<td>2.23±0.30</td>
<td>0/9</td>
</tr>
<tr>
<td>VI</td>
<td>Diltiazem</td>
<td>6.3±0.5***</td>
<td>1.83±0.30</td>
<td>0/9</td>
</tr>
<tr>
<td>VII</td>
<td>Amlodipine</td>
<td>6.3±0.5***</td>
<td>1.66±0.33</td>
<td>0/9</td>
</tr>
<tr>
<td>VIII</td>
<td>BQ + verapamil</td>
<td>4.8±0.4</td>
<td>2.04±0.25</td>
<td>0/9</td>
</tr>
<tr>
<td>IX</td>
<td>BQ + diltiazem</td>
<td>4.0±0.3***</td>
<td>1.66±0.21</td>
<td>0/9</td>
</tr>
<tr>
<td>X</td>
<td>BQ + amlodipine</td>
<td>3.3±0.4***</td>
<td>0.83±0.16*</td>
<td>0/9</td>
</tr>
<tr>
<td>XI</td>
<td>BQT + verapamil</td>
<td>9.6±0.8</td>
<td>1.66±0.33</td>
<td>2/11</td>
</tr>
<tr>
<td>XII</td>
<td>BQT + diltiazem</td>
<td>6.8±0.1***</td>
<td>2.04±0.25</td>
<td>0/9</td>
</tr>
<tr>
<td>XIII</td>
<td>BQT + amlodipine</td>
<td>6.0±0.5***</td>
<td>1.83±0.30</td>
<td>0/9</td>
</tr>
</tbody>
</table>

P values: *<0.05; **<0.01; ***<0.001
*a, ***, ***: compared to control group; †: control group as normal; ‡: compared to corresponding calcium channel blocker alone.
BQ = Betal quid; BQT = Betal quid with tobacco
In the ISO myocardial necrosis model, BQ and BQT were found to interact significantly with amlodipine compared to verapamil and diltiazem. Administration of amlodipine to mice pretreated with BQ or BQT produced a significant alteration in the marker enzyme levels; CK-MB and LDH in both serum and heart homogenate.

Synthetic β-adrenoceptor agonist isoproterenol (ISO) induced myocardial necrosis showed membrane permeability alterations, which bring about the loss of function and integrity of myocardial membrane with development of cardiac hypertrophy as a consequence of increased heart work. Prophylactic administration of BQ with or without amlodipine and diltiazem causes substantial protection from toxic manifestation of ISO. This is demonstrated by fall in specific stages of myocardial necrosis such as interstitial space, infiltration of leucocyte, nuclear duplication and myocyte size.

ISO is a non-specific β-agonist and stimulates all the three types of β-adrenergic receptors. The myocardial necrosis is produced due to its action on the cardiac β₁-receptors. Stimulation of β₁ receptors induced thermogenesis. Hence, after administration of ISO, the animals were maintained under cold conditions to prevent death of the animals due to hyperthermia and respiratory failure. Different mechanisms have been proposed for the action of ISO such as myocardial hypoperfusion, glycogen depletion, electrolyte imbalance, lipid accumulation, lipid peroxidation and free radical damage. Calcium channel antagonists are known to prevent ISO induced myocardial necrosis.
Based on earlier reports on the BQ, it was predicted that both BQ and BQT would increase the ISO induced myocardial damage. Ironically, it was found that BQ had showed a significant protective effect. The exact mechanism by which BQ protected the heart from ISO induced damage cannot be explained. It is speculated that the antioxidant effect of BQ may at least be partly responsible for protecting the heart from ISO induced injury. Further, area canut a major composition of BQ contains arecoline, which is reported to have parasympathomimetic activity that may counteract the increased heart rate produced by ISO and thereby reduce the myocardial hyperperfusion. Calcium, a component of BQ and BQT is necessary for lipolysis that is required for inducing myocardial damage. Betel quid was effective in reducing the injury inspite of calcium overload. The exact reason for this effect cannot be explained with the present data. The administration of BQT aggravated the ISO induced damage. This effect was predicted, as tobacco is known to generate free radicals and to induce heart damage. Further, nicotine one of the component of tobacco is reported to have sympathetic activity, which may also contribute to the cardiac damage induced by BQT.

The results of the present study are contradictory to the earlier reports that BQ chewing induced cardiovascular complications. In the present study, BQ showed cardioprotective effect while BQT aggravated the ISO induced damage. The above results indicate that at least in experimental models, BQ has protective action and BQT induced cardiac damage. It is difficult to predict whether a similar effect will be observed in humans. However, it is worth-mentioning here that some of compounds that showed cardioprotective effect in rats were shown to induce cardiovascular complications in humans, the best example is that of rofecoxib. From the finding of the present study, it can be suggested that studies on the effect of different components on BQ and BQT; betel leaf, arecanut, slaked lime and tobacco should be carried out before concluding the effect of BQ and BQT on cardiovascular system. The present study also indicates that combination of BQ and amlopidine produces additive effect and amlodianpine failed to reverse the BQT induced heart damage.

References
19 Gah JY, Chuang LY & Chen HC, Betel-quad use is associated with the risk of the metabolic syndrome in adults, Am J Clin Nutr, 83 (2006) 1313
34 Vogel HG, Drug discovery and evaluation.2nd edn. (Pringer-Verlag, Berlin, Germany) 2002, p 236
38 Aslam M, Aspects of Asian medicine and its practice in the west, in Trease & Evans pharmacognosy, edited by WC Evans (W.B. Saunders, India) 2002, 469.
40 Kuroda H, Ishiguro S & Mori T, Optimal calcium concentrations in the initial reperfusate for post-ischemic myocardial performance (calcium concentration during reperfusion), J Mol Cell Cardiol, 18 (1986) 625.
43 Wu R, Laplante MA & De Champlain JD, Cyclooxygenase-2 inhibitors attenuate angiotensin II-induced oxidative stress, hypertension, and cardiac hypertrophy in rats, Hypertension, 45 (2005) 1139.