THYMOQUINONE RELAXES INTESTINAL SMOOTH MUSCLES THROUGH MUSCARINIC RECEPTOR AND Ca-CHANNEL BLOCKADE

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ABSTRACT
Background: Functional bowel disorders are common clinical disorders with poorly understood pathophysiology and no gold standard treatment yet. Nigella sativa and its active component thymoquinone are known for their different beneficial medicinal properties and are being widely researched nowadays. Their effect on intestinal motility may prove beneficial in treatment of functional bowel disorders.

Objectives: To study the effect of Thymoquinone on intestinal motility.

Materials & Methods: Rabbits of 1-1.5 kg were sacrificed and effect of thymoquinone was studied on spontaneous contractions in isolated jejunal pieces. Isolated pieces of ileum were used to study contractions induced by carbachol and KCl both alone and in presence of three increasing concentrations of thymoquinone.

Results: Thymoquinone depressed both spontaneous jejunal contractions and contractions induced by carbachol and KCl. The effect was concentration dependant for low and moderate concentrations of thymoquinone on carbachol induced contractions and for moderate and high concentrations of thymoquinone on KCl induced contractions.

Conclusion: Thymoquinone depresses intestinal motility and the possible mechanism may be through muscarinic receptor and Ca-channel blockade. Further studies are needed to establish dose and safety profile.

Keywords: Thymoquinone, intestinal motility, carbachol, KCl.

INTRODUCTION
Gut motility disorders are amongst some of most commonly encountered clinical problems. They range from simple diarrhoea to inflammatory bowel diseases with gross structural changes. A major part of this group are functional bowel disorders (FBD) where primary abnormality is an altered physiological response instead of any established or visible structural or biochemical cause1. One commonly known FBD is Irritable bowel syndrome (IBS) with diarrhoea as a main clinical symptom. It is defined as a FBD characterised by changes in bowel habits associated with abdominal discomfort or overt pain2. Little is understood about its etiology and different mechanisms have been proposed including stress, food allergies, intestinal infection, inflammation, visceral hypersensitivity, genetic transmission, and autoimmune mechanisms3. The FBDs have a high prevalence and affect about 20% of adults in Asia4.
As causative factors remain obscure and pathogenesis is unidentified, no ‘gold standard’ treatments are available5 and usual medical care comprises of patient education, lifestyle changes, antidepressants, antispasmodics, anti diarrheal, and laxatives6. These treatments are only symptom based and improvement is only modest7. Therefore, new drugs merit research and agents with multidimensional therapeutic effects may prove to be more beneficial8.
Nigella sativa is an herbal seed commonly known as ‘black cumin’, ‘kalwanji’ or ‘siyah dana’ belonging to Ranunculaceae family9. It has long been used in traditional medicine for gastrointestinal and airway diseases10. Nigella sativa is being widely researched nowadays and is said to have antibacterial, antifungal, anti-schistosomiasis, antioxidant, antidiabetic, anticancer, anti-inflammatory, analgesic, immunomodulatory, cardioprotective, hepatoprotective, nephroprotective, anti-asthmatic, anticonvulsant and anti-diarrhoeal properties11,12. Thymoquinone is a monoterpenoid hydrocarbon found in Nigella sativa13 and the anti-diarrhoeal property is attributed to it as the milk extraction of Nigella sativa which lacks thymoquinone does not inhibit diarrhoea12.
In this study we have explored the effect and possible mechanism of action of thymoquinone on intestinal motility as it may become a useful agent for FBDs to control this poorly understood and still not well treated clinical entity.
MATERIALS AND METHODS

Animals
Rabbits of both genders weighing 1-1.5 kg were purchased from local market and kept in animal house of PGMI. They were deprived of food but not water 14 hours prior to experiment. They were sacrificed in Pharmacology laboratory of PGMI.

Isolated Tissue Preparation
Abdomen was opened, jejunum and ileum were identified, excised and put in Tyrode’s solution. Mesentery was cleaned and about 2 cm long segments were mounted in 50 ml organ bath containing Tyrode’s solution maintained at 37°C and aerated with O₂. Composition of Tyrode’s solution was NaCl (8.0 gm/L), KCl (0.2 gm/L), CaCl₂ (0.2 gm/L), MgCl₂ (0.1 gm/L), NaH₂PO₄ (0.05 gm/L), NaHCO₃ (1.0 gm/L), Dextrose (1 gm/L) and it was freshly prepared. Tissues were allowed to equilibrate for 30 minutes before addition of any drug. Intestinal contractions were recorded isotonically using Harvard kymograph.

Chemicals and drugs
Thymoquinone (Aldrich) dissolved in 50% ethanol. Carbachol (Alfa Aeser) dissolved in distilled water. Potassium chloride (Merck) dissolved in distilled water. Chemicals for Tyrode’s solution: NaCl (Merck), KCl (Merck), CaCl₂ (Merck), MgCl₂ (Merck), NaH₂PO₄ (Merck), NaHCO₃ (Merck), Dextrose (Merck).

Experiment
Effect of thymoquinone on jejunal contractions (n=7)
Normal spontaneous jejunal contractions were recorded for 30 seconds and then thymoquinone was added to the organ bath in cumulative fashion in concentrations ranging from 3.04 µM to 97.28 µM. Effect of each concentration was recorded on a graph paper for 30 seconds.

Effect of thymoquinone on Carbachol induced contractions (n=6)
Normal spontaneous ileal contractions were recorded for 30 seconds and then carbachol was added to the organ bath in cumulative fashion in concentrations ranging from 0.55 nM to 17.6 nM. After three times washing and giving rest to the tissue for 30 minutes, the same experiment was repeated in presence of three increasing concentrations (3.04, 6.08, and 12.16 µM) of thymoquinone. Tissue was washed three times and rest of 30 minutes was given after each experiment.

Effect of 50% ethanol on jejunal contractions
As alcohol was used as a solvent for thymoquinone, effect of increasing concentrations of ethanol was also studied on jejunal contractions. It did not produce any effect on spontaneous jejunal contractions.

STATISTICAL ANALYSIS
Mean ± SEM was calculated for each response. Log concentration response curves were plotted. Responses to agonists in absence and presence of thymoquinone were compared for by using two tailed Student’s t-test for paired samples. p-value ≤ 0.05 was level of significance.

RESULTS
Effect of thymoquinone on jejunal contractions
The spasmolytic effect was concentration dependant with complete inhibition of contractions (Figure 1). EC₅₀ value was 40 µM (Figure 2).

Effect of thymoquinone on Carbachol induced contractions
Pre-treatment with thymoquinone prevented carbachol induced contractions of rabbit ileum in a concentration dependant manner. There was rightward shift of carbachol concentration response curve in presence of thymoquinone (Figure 3). Difference was significant between carbachol alone and in presence of low concentration of thymoquinone as well as between low concentration and medium concentration of thymoquinone. However difference between medium and high concentrations of thymoquinone was not significant.

Figure no. 1: Effect of thymoquinone on spontaneous contractions of rabbit jejunum.
Effect of thymoquinone on KCl induced contractions

Pre-treatment with thymoquinone produced rightward nonparallel shift of KCl concentration response curves on rabbit ileum (Figure 4). Difference was significant between KCl alone and in presence of medium concentration of thymoquinone. There was no significant difference between KCl alone and in presence of lower concentration of thymoquinone and between medium and higher concentration of thymoquinone.

DISCUSSION

We designed this experiment to confirm whether thymoquinone relaxes intestinal smooth muscle and can it decrease intestinal motility and it has exhibited intestinal smooth muscle relaxant properties in this study.

The rabbit jejunum was selected to study the direct relaxant properties of thymoquinone on intestine because jejunum is known for its prominent spontaneous contractions which are easy to record and any effect that decreases them can be demonstrated and studied legibly. In this experiment thymoquinone has decreased both the frequency and height of spontaneous jejunal contractions in a concentration dependant fashion. Higher doses of thymoquinone have almost abolished jejunal activity. EC50 for thymoquinone was 40 µM.Ghayur et al14 also conducted a similar study on rabbit jejunal preparation and demonstrated spasmolytic activity of thymoquinone with EC50 of 156.4 µM. Gilani, et al.15 studied effect of Nigella sativa crude extract on spontaneous contraction of rabbit jejunum with similar results. In another study, intestinal transit in rats was estimated and it was found to be slowed down in rats receiving aqueous extract of Nigella sativa with p value < 0.05 as compared to control group16.

In an intact individual, gastrointestinal motility is controlled by many mediators like acetylcholine, histamine, serotonin, opioids and the

Figure 4: Graph showing effect of KCl alone and in presence of three increasing concentrations of thymoquinone on rabbit ileal motility. Maximum height of contraction induced by KCl is taken as 100%.
* p-value ≤ 0.05 vs KCl alone.
** p-value ≤ 0.01 vs KCl alone.
*** p-value ≤ 0.001 vs KCl alone.
^ p-value ≤ 0.05 vs KCl and thymoquinone 3.04 µM.
^^ p-value ≤ 0.01 vs KCl and thymoquinone 6.08 µM.
respecte receptors\textsuperscript{17}. All these receptors have different chemical and signalling pathways but the final step in exerting smooth muscle contraction is an increase of calcium concentration in smooth muscle cells through activation of L-type Ca-channels resulting in coupling of myosin and actin producing shortening of muscle fibres. Any agent that blocks these Ca-channels will, therefore, relax smooth muscles whatever the mediator may be\textsuperscript{18}.

To demonstrate the effect of thymoquinone on different receptors and hence its possible mechanism of action, we devised two sets of experiments. Rabbit ileum was chosen for these experiments because it has maximum density of receptors\textsuperscript{19} and agonist-induced contractions are easy to be elaborated. In first set, we chose carbachol as the agonist of muscarinic receptors. Carbachol was selected instead of acetylcholine due to its chemical stability to hydrolysis by acetylcholinesterase\textsuperscript{20} thus making it possible to record cumulative concentration responses. A cumulative concentration response curve was plotted for carbachol and then repeated in presence of three increasing concentrations of thymoquinone. Thymoquinone depressed the effect of carbachol on rabbit ileum. This depressant effect was also concentration dependent. The effect was significant with lower (3.04 \mu M) and moderate (6.08 \mu M) concentrations of thymoquinone (p-value ≤ 0.05). The difference between moderate and high concentrations (12.16 \mu M) of thymoquinone was not significant. In no study effect of any preparation of Nigella sativa or thymoquinone was studied on muscarinic receptors in intestine. But studies on other smooth muscle preparations demonstrate antimuscarinic property of Nigella sativa extracts as well as thymoquinone\textsuperscript{14,21,22,23}.

In the second set of experiments, KCl was used as an agonist of rabbit ileum motility. KCl produces high concentrations of K\textsuperscript{+} in solution/extracellular environment that causes abrupt opening of Ca\textsuperscript{2+} channels and smooth muscle contraction\textsuperscript{18}. First a cumulative concentration response curve was plotted with KCl and then the experiment was repeated in presence of three increasing concentrations of thymoquinone. The KCl induced ileal contractions were depressed more significantly by moderate concentration (6.08 \mu M) of thymoquinone (p-value ≤ 0.05). There was no significant depression with low concentration (3.04 \mu M) of thymoquinone and difference between moderate and high concentrations of thymoquinone was only significant at higher doses of KCl (p-value <0.05). This effect of thymoquinone on Ca-channels of intestinal preparations was also demonstrated in another studies using verapamil as standard calcium channel blocker(14). Calcium channel blockade by Nigella sativa was also demonstrated on other smooth muscle preparations(15, 24) and myocardium\textsuperscript{25,26}.

**CONCLUSION**

The mechanism of intestinal smooth muscle relaxant effect of thymoquinone is multidimensional through more than one receptor. Thymoquinone acts through muscarinic receptors at low concentration and through Ca\textsuperscript{2+}-channels blockade at higher concentrations. However, further studies are needed to establish dose and safety profile.

**REFERENCES**