Original Article

THYMOL TEMPERS As (III) AND Hg (II) CAUSED HYPERCONTRACTILITY BY SIMILAR PATHWAYS IN ISOLATED AORTIC AND TRACHEAL RINGS

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ABSTRACT

Objective: Thymol is known to cause smooth muscle relaxation, possibly by inhibition of Ca^{2+} influx, release or loss of its sensitivity. It is, however, also reported to be a strong antioxidant in several other systems. In this study, we investigate other possible pathways of thymol caused relaxation of aortic and tracheal segments by using specific blockers, and explore it as potential ameliorator of arsenic and mercury caused hyper contraction.

Methods: Male Wistar rats were used in all experiments. Aorta/trachea was carefully removed, cleaned, and cut into 2-mm thick rings. Isometric contractions were measured using organ bath system. One-way analysis of variance (ANOVA) and Student's *t*-test were used for statistical analyses. $P<0.05$ was considered significant.

Results: In pollutant unexposed aortic and tracheal segments, thymol is found to inhibit contractions through quenching of reactive oxygen species (ROS) in addition to its previously reported effects on Ca^{2+} movements. Equal effectiveness in absence and presence of N^{G}-nitro-L-arginine methyl ester (L-NAME) indicates that nitric oxide (NO) has no significant role in the thymol caused relaxation.

Conclusion: In both muscle types, thymol is found to be an effective ameliorator of As (III) and Hg (II) caused hyper contraction, at low concentrations; it acts by inhibiting the Ca^{2+} influx whereas at high concentration, it acts by blocking Ca^{2+} influx and neutralizing ROS.

Keywords: Arsenic, Aorta, Mercury, Trachea, Thymol, ROS

INTRODUCTION

Arsenic and mercury continue to be among top three toxic metals worldwide as per Agency for Toxic Substance and Drug Registry [1]. Current estimates suggest that global emissions of arsenic and mercury and population coming in contact with them continue to increase [2, 3]. Acute and chronic exposures to As (III) and Hg (II) are known to affect smooth muscles of cardiovascular and respiratory systems [4, 5]. Earlier studies, including our own, have shown that toxic effect of As (III) and mercury Hg (II) on smooth muscles is mediated through the reactive oxygen species (ROS) generation and nitric oxide (NO) depletion [6-9]. Molecules which modulate production of these signaling entities can, therefore, be investigated for their ameliorative effect on As (III), and Hg (II) caused smooth muscle hyper-contraction. In this regard, thymol (2-isopropyl-5-methylphenol), a natural phenol derivative of cymene, has been reported to have antispasmodic action on skeletal, smooth and cardiac muscles [10]. Thymol has been shown to inhibit contraction induced by various spasmogen in the aorta, ileum, uterus and smooth duodenum muscles and thus causes relaxation [11-13]. The exact mechanism of thymol caused relaxation of pre-contracted muscle is not known, but inhibition of internal Ca^{2+} release and/or loss of Ca^{2+} sensitivity are suggested as possible routes by various investigators [12, 14]. The effect of thymol on the tracheal system is largely unexplored.

In this study, we investigate the effect of thymol on resting tone and agonist-induced contraction of aortic and tracheal smooth muscles. Mechanism of thymol caused alteration in smooth muscle contraction is investigated by using specific blockers of other contractile pathways in addition to Ca^{2+} influx. Moreover, thymol is explored as a possible ameliorator of As (III) and Hg (II)-induced hyper contraction in aortic and tracheal tissues.

MATERIALS AND METHODS

Chemicals

Acetylcholine (ACh), apocynin, arsenic (As $(NO_2)_3$), bradykinin, mercury $(HgCl_2)$, N^{G}-nitro-L-arginine methyl ester (L-NAME), phenylephrine (PE), and thymol were obtained from Sigma-Aldrich (USA). All inorganic chemicals were sourced from Merck (India). Thymol were first dissolved in 10% dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich (USA) and diluted. Final vehicle concentration, to which tissues were exposed, was 0.1%. At this concentration DMSO did not influence PE/ACh-induced contraction [6].

Animals and Isometric tension measurement

Animals were maintained according to recommendations of the U. S. Guide for the Care and Use of Laboratory Animals in the National Institute of Health and the study was approved by Institutional Animal Ethical Committee (IAEC, no. 004/2013), Jamia Millia Islamia (New Delhi, India). Male Wistar rats, weighing between 300-500 g, were anesthetized and sacrificed by cervical dislocation [15]. Thoracic aortae and tracheae were dissected, cleaned off fats and adhering tissues, and cut into rings of approximately 2-3 mm in length. Rings mounted between force transducers and rigid support of the organ bath system (MLT0420, AD Instruments, Australia) were super fused with Krebs-Henseleit solution (in mM: NaCl (120), $NaHCO_3$ (25), $MgSO_4$ (1.2), KH_2PO_4 (1.2), KCl (4.72), $CaCl_2$ (2.5) and $C_6H_{12}O_6$ (11)), pH 7.4, 37 ^{0}C, gassed with 95% O_2 and 5% CO_2. Isometric force was recorded on digital acquisition and analysis system (Power Lab 8/35, AD instruments, Australia). Passive tension was adjusted to 2 g over 60 min equilibration periods with frequent changes of buffer until a stable baseline tension was obtained. Integrity of aortic endothelium and tracheal epithelium was assessed with 1 μ mol/l ACh and 1 μ mol/l bradykinin, respectively. At the end of the equilibration period, aortic and tracheal rings were contracted with 1 μ mol/l PE or 1 μ mol/l ACh. Aortic/tracheal rings were then washed several times with buffer and allowed to equilibrate for 30 min before eliciting agonist-induced contraction.

Experimental protocol

The effect of thymol on resting tone and agonist-induced contraction was assessed by incubating aortic and tracheal rings with varying concentrations of thymol for 40 min. To delineate the contractile pathway affected by thymol, aortic or tracheal rings were pre-

incubated with apocynin (100 μ mol/l), verapamil (1 μ mol/l) or L-NAME (30 μ mol/l) for 40 min following which thymol exposure was given for another 40 min. The ameliorative effect of thymol on metal exposed rings was investigated by co-incubating rings with thymol and As(III) or Hg(II) for 40 min. Concentrations and incubation periods of As (III) and Hg (II) are based on previous findings of our laboratory [6, 7]. Incubation of aorta/trachea with As (III), Hg (II), apocynin, verapamil, or L-NAME alone did not cause any significant change in resting tension in the absence of agonists.

Statistical analysis

Each response was tested on 5 aortic/tracheal rings taken from different rats, and was compared with control rings from the same animal and variation is reported in percent terms. Data are expressed as mean±standard error of mean (S. E. M). The significance ($P \leq 0.05$) of the results was assessed by means of unpaired Student's *t*-tests, and one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

RESULTS

Fig. 1 shows thymol caused variation in resting tension of aortic and tracheal rings in the absence of any agonist. Rings with an applied tension of 2.0 g did not show any significant change when incubated with thymol up to 100 μ mol/l. Further increase in thymol concentration led to a significant increase in resting tension which was observed to be 15% for the aorta and 18% for trachea at 200 μ mol/l thymol.
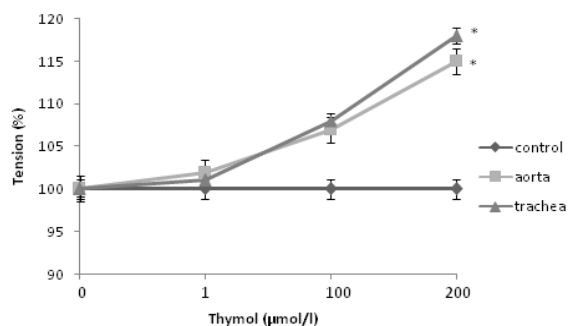
Fig. 1: Effect of thymol on resting tension of aortic and tracheal rings in the absence of agonists. Results are shown as mean±S. E. M.; *n* = 5; *P<0.05, thymol vs. Control (ANOVA followed by Duncan's multiple range tests)
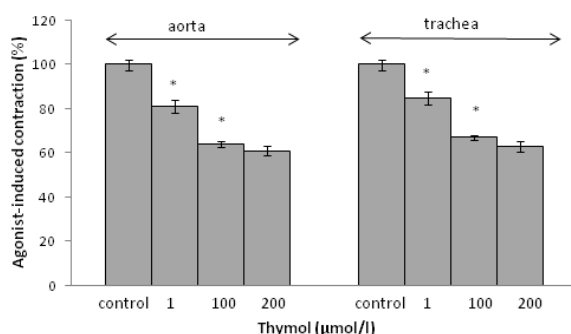

Fig. 2: Inhibitory effects of varying concentrations of thymol on PE and ACh-induced contractions in rat aortic and tracheal rings. Results are presented as mean±S. E. M.; *n* = 5; *P<0.05, 1 μ mol/l vs. Control; 100 μ mol/l vs. 1 μ mol/l, 200 μ mol/l vs. 100 μ mol/l (one-way ANOVA followed by Duncan's multiple range test).

Agonist-induced contractile responses decreased, both for aortic and tracheal rings in the presence of thymol (fig. 2). PE-induced aortic contraction and ACh-induced tracheal contraction was significantly inhibited up to 100 μ mol/l thymol concentrations. The saturating effect was seen beyond this concentration of thymol. Since 200 μ

mol/l thymol caused a non-specific increase in the muscle tone, this is coupled with the fact that no significant difference was seen in the relaxation magnitude between 100 μ mol/l and 200 μ mol/l. This suggested that optimal thymol concentration for delineation of the relaxation pathway was 100 μ mol/l. We have previously reported saturating effect producing a concentration of apocynin, verapamil and L-NAME in aortic and tracheal systems as 100 μ mol/l, 1 μ mol/l and 30 μ mol/l, respectively [6, 7, 16, 17].

To assess the potential relaxation pathways of thymol, aortic/ tracheal rings were incubated with saturating effect producing a concentration of thymol alone or in combination with three modulators (table 1).
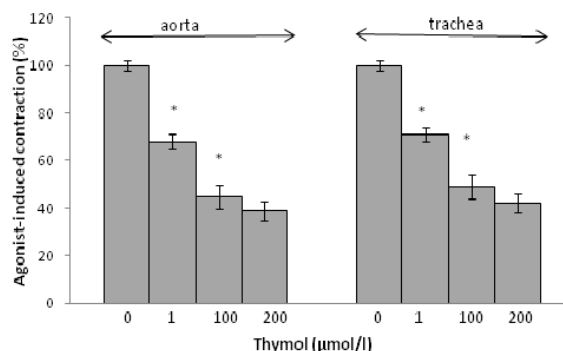
Fig. 3A: Effects of thymol on contraction induced by PE/ACh in rat aortic/tracheal rings pre-exposed to 25 μ mol/l As (III). The presence of arsenic caused hyper contraction of 32% and 24% in aorta and trachea, respectively. Results are shown as mean±SEM; #P<0.05, unpaired Students *t*-test, 1μmol/l vs. No thymol; 100 μ mol/l vs. 1 μ mol/l, 200 μ mol/l vs. 100 μ mol/l
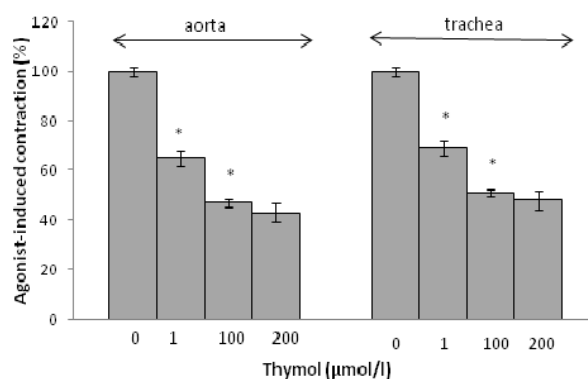
Fig. 3B: Effects of thymol on contraction induced by PE/ACh in rat aortic/ tracheal rings pre-exposed to 6 nmol/l Hg(II). The presence of mercury caused hyper contraction of 34% and 62% in aorta and trachea, respectively. Results are shown as mean ± S.E.M.; #P < 0.05, unpaired Students *t*- test, 1μmol/l vs. No thymol; 100 μmol/l vs. 1 μmol/l, 200 μmol/l vs. 100 μmol/l

Table 1: Inhibitory effect of thymol on contraction induced by PE (aorta) and ACh (trachea) in the presence of apocynin, verapamil, and L-NAME

	Aorta (% contraction)	Trachea (% contraction)
Control	100±2.4	100±1.5
Thymol	64±1.6*	67±2.0*
Apocynin+Thymol	63±3.9	65±2.7
Verapamil+Thymol	56±4.2#	52±1.3#
L-NAME+Thymol	70±3.6	75±2.9

Results are presented as mean±S. E. M.; *n* = 5; *P<0.05, one-way ANOVA followed by Duncan's multiple range test, thymol vs. control and #P<0.05, unpaired Students *t*-test, thymol+modulators vs. thymol

Compared to thymol alone, co-incubation of aortic or tracheal rings with ROS inhibitor (apocynin) did not cause any significant change in agonist-induced contraction. Co-incubation of both types of rings with Ca^{2+} channel blocker (verapamil) and thymol, however, led to significant decrease in agonist-induced contraction. NO is a major smooth muscle relaxant [18], rings when incubated with L-NAME (an inhibitor of NO synthase) showed an increased contraction of 9% in the aorta and 13% in the trachea. The magnitude of contraction showed by both the rings in the presence of thymol and L-NAME, however, showed no significant change when compared to thymol alone.

Both, As (III) and Hg (II), are reported to cause increased contraction of aortic and tracheal smooth muscle, which appears to originate from increased ROS generation, increased Ca^{2+}influx or NO depletion [6-9]. Aortic and tracheal rings exposed to As (III) (25 μ mol/l) caused an increased contractile response by 32% and 24% when induced with PE or ACh, respectively. This increased response taken as 100% has been compared with magnitudes obtained when rings were co-incubated with thymol (fig. 3. A). Thymol was found to be more effective in suppressing contraction in As(III) exposed rings as compared to unexposed rings. This increased effectiveness at 1 μ mol/l thymol was 13% for aortic and 14% for tracheal rings, at 100 μ mol/l thymol this difference was 19% and 18%, respectively. No significant change was observed at 200 μ mol/l thymol when compared to 100 μ mol/l thymol for As (III)-exposed aortic or tracheal rings. Similar trends were seen for Hg (II) exposed aortic and tracheal rings (fig. 3. B), thymol was more effective in suppressing contractions in Hg (II) hyper contracted rings as compared to unexposed rings. At 1 μ mol/l and 100 μ mol/l thymol, this increased suppression was 16% and 17% for aortic rings, and 16% and 15% for tracheal rings.

DISCUSSION

In the absence of agonist, resting tension of aortic smooth muscle is reported to increase at high thymol concentrations. This effect is reported to arise from the release of Ca^{2+} from sarcoplasmic reticulum [19-21] and is not a part of the normal contraction cascade. In the presence of agonists (PE/ACh), however, normal cascade operates, and effect of thymol can be seen at low concentrations which arise from its interaction with components of normal cascades which leads to relaxation. Effect of thymol in the absence of agonists obtained in our present study is in concordance with the earlier studies suggesting an increase in resting tension on incubating smooth aortic muscles with high (200 μ mol/l) thymol concentration [12]. A similar trend was obtained for tracheal rings where no change in resting tension was seen up to 100 μ mol/l thymol indicating that both the smooth muscles respond similarly to thymol in the absence of agonists. In the presence of agonist thymol, however, has shown inhibition of contraction in both aortic and tracheal rings. Aortic and ileal smooth muscles have been reported to show relaxation in the presence of thymol possibly through inhibition of intracellular Ca^{2+}release [12, 21], in this study, we show that tracheal smooth muscles follow the same pattern of relaxation as that of the aorta. In addition to direct stimulation of intracellular Ca^{2+}release, agonist-induced contraction of smooth muscle has contributed from ROS, NO, and extracellular Ca^{2+} influx [22-24]. Quenching of ROS by apocynin and prevention of extracellular Ca^{2+} influx by blocking L-type voltage-dependent calcium channel (VDCC) through verapamil is known to decrease contractile response [6, 25, 26].

Co-incubation of aortic and tracheal rings with thymol and apocynin decreased contractile response by the same magnitude as that by thymol alone. Apocynin directly scavenges ROS in vascular cells [26], it also inhibits superoxide anion generation by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) [27]. With respect to our results, however, both actions of apocynin lead to the same effect, i.e. decrease in ROS signaling contraction suggesting that thymol and apocynin act on the same pathway i.e. ROS quenching. Co-incubation of rings with verapamil and thymol, however, leads to significant decrease in contraction magnitude as compared to thymol alone. Since saturating verapamil concentration is employed, it indicates that thymol acts on other pathways also in addition to Ca^{2+}homeostatsis as suggested by other investigators [12]. This additional route is possibly ROS quenching and not NO based because thymol is found to be equally effective in the presence and the absence of L-NAME. Other investigators have also reported that the thymol caused relaxation in smooth muscle is NO-independent [12, 28].

As (III) and Hg (II) are reported to cause hyper contraction of smooth muscles through pathways involving ROS generation, NO depletion, and Ca^{2+} influx. Since in this study thymol shows significant relaxation of both aortic and tracheal smooth muscles acting through ROS quenching in addition to its reported effect on Ca^{2+}influx, release and sensitivity, we explore it as a possible ameliorator of As(III) and Hg(II) caused hyper contraction in both smooth muscle systems. Aortic rings exposed to As (III) and Hg (II) was more effectively inhibited by thymol (table 2. A and 2. B). Low thymol concentrations are known to affect the only Ca^{2+}influx whereas high concentration inhibits intracellular Ca^{2+}release and sensitivity [12]. In muscle unexposed to either of the metals, saturation in relaxant response was observed at high thymol concentration (100 μ mol/l) for both aorta and trachea suggesting that ROS and intracellular Ca^{2+}release is effectively inhibited only at high thymol concentration. Increased relaxation, even at low thymol concentration (1 μ mol/l) was seen for both As (III)-exposed smooth muscles which can be attributed to increased Ca^{2+}influx in the presence of As (III). Higher relaxation in As (III)-exposed muscles at 100 μ mol/l thymol, indicates ROS increase, which is quenched by high thymol concentration. Similar trends are obtained for mercury exposed aortic and tracheal rings, suggesting an increase of Ca^{2+}influx and rise in ROS as possible modes of hyper contraction. Thymol is thus found to be an effective ameliorator of As (III) and Hg (II) exposed aortic and tracheal smooth muscles which at low concentrations inhibit only Ca^{2+} influx. It is more effective ameliorate at higher concentrations as it also neutralizes ROS in addition to inhibition of Ca^{2+}influx.

Table 2A: Mean relaxation (%) of unexposed and As/Hg exposed aortic rings caused by various thymol concentrations. Results are showed as mean±SEM; *n* = 5; *$P<0.05$, one-way ANOVA followed by Duncan's multiple range test, As(III)/Hg(II) exposed vs. unexposed for same concentration; #$P<0.05$: unpaired Students *t*-test, 1μmol/l vs. control; 100 μ mol/l vs. 1 μ mol/l, 200 μ mol/l vs.100 μ mol/l

Aorta	1 μmol/l	100 μmol/l	200 μmol/l
Unexposed	19±2.8#	36±1.7#	39±3.1
As(III)-exposed	32±2.1*,#	55±3.6*, #	61±2.9*
Hg(II)-exposed	35±3.0*, #	53±1.2*, #	57±3.6*

Table 2B: Mean relaxation (%) of unexposed and As /Hg exposed tracheal rings caused by various thymol concentrations. Results are showed as mean±SEM; *n* = 5; *$P<0.05$, one-way ANOVA followed by Duncan's multiple range test, As(III)/Hg(II) exposed vs. unexposed for same concentration; #$P<0.05$: unpaired Students *t*-test, 1μmol/l vs. control; 100 μ mol/l vs. 1 μ mol/l, 200 μ mol/l vs.100 μ mol/l

Trachea	1 μmol/l	100 μmol/l	200 μmol/l
Unexposed	15±2.3#	33±2.2#	37±3.4
As(III)-exposed	29±1.9*,#	51±3.9*,#	58±3.0*
Hg(II)-exposed	31±2.7*,#	48±1.1*,#	52±3.7*

CONCLUSION

In pollutant unexposed aortic and tracheal rings, thymol is found to inhibit contractions through quenching of ROS in addition to its previously reported effects on Ca^{2+} movement. NO has no significant role in the thymol caused relaxation. Thymol is found to be an ameliorator of As (III) and Hg (II) caused hyper contraction of both muscle types; it appears to act by inhibiting Ca^{2+} influx only at low concentrations. At high concentrations, where it found to be more effective ameliorator, it also neutralizes ROS in addition to inhibiting Ca^{2+} influx.

ABBREVIATION

ACh, aacetylcholines; DMSO, dimethyl sulfoxide; L-NAME, N^G-nitro-L-arginine methyl ester; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NO, nitric oxide; PE, phenylephrine; ROS, reactive oxygen species; VDCC, voltage-dependent calcium channel.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

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