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RAPID COMMUNICATION

Inhibitory effects of genistein and resveratrol on guinea pig gallbladder contractility *in vitro*

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Abstract

AIM: To observe and compare the effects of phytoestrogen genistein, resveratrol and 17β-estradiol on the tonic contraction and the phasic contraction of isolated gallbladder muscle strips and to study the underlying mechanisms.

METHODS: Isolated strips of gallbladder muscle from guinea pigs were suspended in organ baths containing Kreb's solution, and the contractilities of strips were measured before and after incubation with genistein, resveratrol and 17β-estradiol respectively.

RESULTS: Similar to 17β-estradiol, genistein and resveratrol could dose-dependently inhibit the phasic contractile activities, they decreased the mean contractile amplitude and the contractile frequencies of gallbladder muscle strips, and also produced a marked reduction in resting tone. The blocker of estrogen receptor ICI 182780 failed to alter the inhibitory effects induced by genistein and resveratrol, but potassium bisperoxo (1, 10 phenanthroline) oxovanadate bpV (phen), a potent protein tyrosine phosphatase inhibitor, markedly attenuated the inhibitory effects induced by genistein and resveratrol. In calcium-free Kreb's solution containing 0.01 mmol/L egtazic acid (EGTA), genistein and resveratrol inhibited the first phasic contraction induced by acetylcholine (ACh), but did not affect the second contraction induced by $CaCl_2$. In addition, genistein, resveratrol and 17β-estradiol also could reduce the contractile responses of ACh and KCl, and shift their cumulative concentration-response curves rightward.

CONCLUSION: Phytoestrogen genistein and resveratrol can directly inhibit the contractile activity of isolated gallbladder muscle both at rest and in response to stimulation. The mechanisms responsible for the inhibitory effects probably due mainly to inhibition of tyrosine kinase, Ca^{2+} influx through potential-dependent calcium channels (PDCs) and Ca^{2+} release from sarcoplasmic reticulum (SR), but were not related to the estrogen receptors.

Key words: Phytoestrogen; Estradiol; Gallbladder; Smooth muscle; Ca^{2+} channel

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INTRODUCTION

Female sex hormones are known to affect cholesterol metabolism, gallbladder and sphincter of Oddi motility[1-4]. Phytoestrogens represent a wide group of compounds which are naturally found in many plants, and they are defined as plant substances that are structurally or functionally similar to estradiol[5,6]. These natural products possess a wide spectrum of physiological and pharmacological effects such as estrogenic effects[7,8], anti-atherosclerosis[9], anti-osteoporosis[10], relieving menopausal symptoms[11] and the inhibitory effect of tyrosine kinases[12,13]. Recently, many papers indicate that phytoestrogen genistein and

resveratrol can inhibit vasocontractile responses and relax vascular smooth muscles by a Ca^{2+} antagonistic property which is very similar to estradiol[14,15], and data from electrophysiological studies suggest genistein reversibly inhibits L-type calcium current in isolated guinea-pig ventricular myocytes in a concentration-dependent manner[16]. Therefore, the biological actions of genistein and resveratrol are very useful for medicine and nutrition, and they are proposed to have potential as natural substitutes of estrogen therapy. Despite the increasing interest in the effects of phytoestrogen in the cardiovascular system, little is known about the effect of genistein and resveratrol on gallbladder smooth muscle. The present study was designed to observe and compare the effects of phytoestrogen genistein, resveratrol and 17β-estradiol on the tonic contraction and the phasic contraction of isolated gallbladder muscle strips both at rest and in response to acetylcholine (ACh) and KCl and to study its underlying mechanisms.

MATERIALS AND METHODS

Animal and tissue preparation

The present work was conducted in conformity with the procedures described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and the procedures performed were in accordance with institutional guidelines. Adult male or non-pregnant female guinea pigs (weighing 387.8 ± 7.5 g, provided by the experimental animal center of Lanzhou University) were utilized in this study. Preliminary studies indicated that no differences existed between the sexes with respect to either the contractile responsiveness to the agonists or to the sensitivities to genistein, resveratrol and 17β-estradiol. All animals were fasted overnight prior to being sacrificed by overdose injection of pentobarbitone and the whole gallbladders were quickly removed and placed in Kreb's solution containing the following compositions (mmol/L): NaCl 120, KCl 5.9, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, $CaCl_2$ 2.5, and glucose 11.5, buffered at pH 7.4. After removal of the mucosa by blunt dissection, muscle strips (5 mm × 10 mm) were prepared from the body of the gallbladder by cutting parallel to the long axis of the tissue, and then were mounted horizontally in separate 5-mL tissue chambers containing 37 ± 0.5℃ Kreb's solution, bubbled with 95% O_2 and 5% CO_2. The muscle preparations were allowed to equilibrate for 2-3 h with a resting tension of 1.0 g and the solution was changed every 20 min. The isometric contractions were measured with force transducers and recorded with the BL-420E$^+$ experimental system of biological function (TME, China) by microcomputer.

Experimental protocols

In order to observe the effects of genistein, resveratrol and 17β-estradiol on the basal contractile activities of gallbladder, the different concentration (1, 10, 20 or 40 μmol/L) of genistein, resveratrol and 17β-estradiol or the same dose of solvent (control) was added respectively to the tissue chamber for 10 min. A specific antagonist of estrogen receptor ICI 182780 (10 μmol/L) or a potent protein tyrosine phosphatase inhibitor bpV (phen) (1 μmol/L) was added 10 min before administration of 10 μmol/L genistein or resveratrol to investigate whether their actions were relevant with the estrogen receptors and tyrosine kinase inhibition in gallbladder smooth muscle.

To evaluate the possible effect of genistein, resveratrol and 17β-estradiol on ACh-induced calcium release and calcium influx through receptor-operated calcium channels (ROCs), gallbladder muscle strips were incubated in calcium-free Kreb's solution containing 0.01 mmol/L egtazic acid (EGTA) for 30 min, and then treated with ACh (10 μmol/L). When the contractile response had reached a plateau, $CaCl_2$ (10 mmol/L) was added into the organ chamber and a further contraction was obtained. Tissues were washed with Ca^{2+}-free Kreb's solution and left to return to baseline tone. The strips were then treated by ACh and $CaCl_2$ again after being incubated with genistein (20 μmol/L) and resveratrol (20 μmol/L) or the same dose of solvent for 6-8 min.

In some experiments, to determine the effect of genistein, resveratrol and 17β-estradiol on contractile response to ACh and potassium, the control contractile response curves to ACh (10^{-8}-10^{-3} mol/L) and KCl (10-100 mmol/L) were obtained respectively, then the strips were washed repeatedly with Kreb's solution until the strips returned to the basal tension. The strips were then incubated with genistein (40 μmol/L), resveratrol (40 μmol/L) or 17β–estradiol (40 μmol/L) for 10 min respectively, and ACh or KCl concentration-dependent contraction curve was obtained again.

Drugs

Genistein, resveratrol, 17β-estradiol (Sigma, Chemical Co, USA); ACh (the Second Pharmaceutical Factory of Beijing, China); ICI 182780 (Tocris Cookson Inc., Bristol, UK); potassium bisperoxo (1,10 phenanthro line)oxovanadate bpV (phen) (Alexis Biochemicals, San Diego, CA). ICI 182780, genistein, resveratrol and 17β-estradiol were dissolved with dimethyl sulphoxide (DMSO). The final concentration of DMSO in the bath in each case was always no more than 0.1% and had no effect on basal contraction.

Statistical analysis

All results are expressed as mean ± SE. "*n*" refers to the number of guinea pigs used in the study. Data were expressed as % decrease in the basal tension, mean amplitude and mean frequencies of phasic contraction. In experiments involving concentration-response curves, the results were expressed as percentage of control maximal contractile responses induced by 10^{-3} mol/L ACh or 100 mmol/L KCl respectively. The EC_{50} value of each strip was determined by the Scott Method, and was expressed as negative log molar (pD_2) value. Statistical analysis was performed using the Student *t*-test and analysis of variance (ANOVA). Each group was compared with the solvent control. $P < 0.05$ was considered significant.

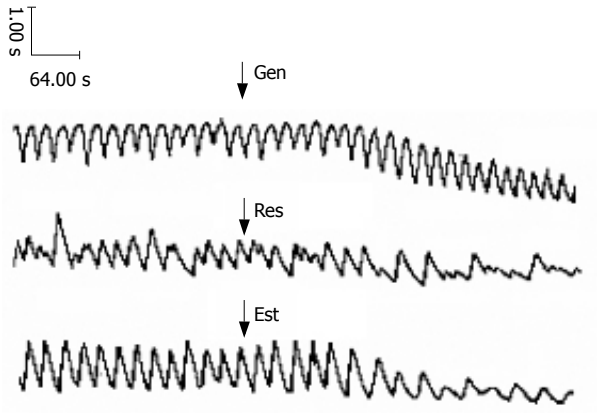

Figure 1 Sample traces showing the basal contractile activity of the gallbladder before and after the administration of 20 μmol/L genistein (Gen), resveratrol (Res) and 17β-estradiol (Est).

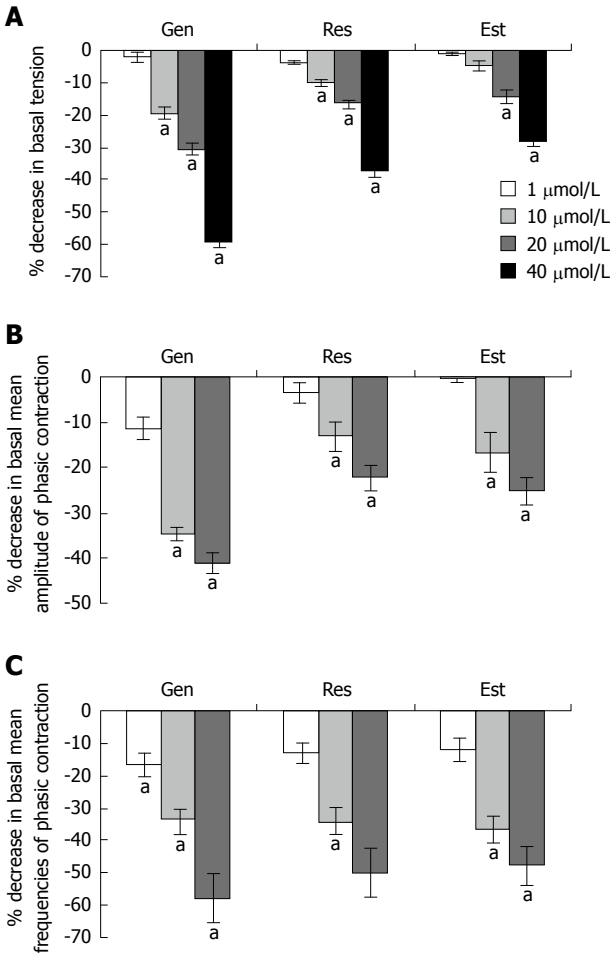

Figure 2 Effects of genistein (Gen), resveratrol (Res) and 17β-estradiol (Est) on resting tension (**A**), mean contractile amplitude (**B**) and (**C**) mean frequencies of phasic contraction in isolated guinea pig gallbladder muscle strips (n = 10). $^aP < 0.05$ *vs* solvent control.

RESULTS

Effects of genistein, resveratrol and 17β-estradiol on basal activities of gallbladder muscle strips

The spontaneous contractile activities of isolated gallbladder smooth muscle were not very regular, and some strips had obvious spontaneous phasic contractions with mean amplitude of 0.49 ± 0.06 g and mean frequencies of 2.80 ± 0.25 waves/min (Figure 1) while the others only possessed tonic contraction. In the strips with spontaneous phasic contractions, genistein, resveratrol and 17β-estradiol (1, 10, 20 or 40 μmol/L) could dose-dependently inhibit the phasic contractile activities, they decreased the mean contractile amplitude and the contractile frequencies and also produced a marked reduction in resting tone (Figures 1 and 2). Increasing the concentrations of the above three estrogens to 40 μmol/L, the phasic contractile activities disappeared completely, the decreased percentages of the mean contractile amplitude and the contractile frequencies all reached 100%.

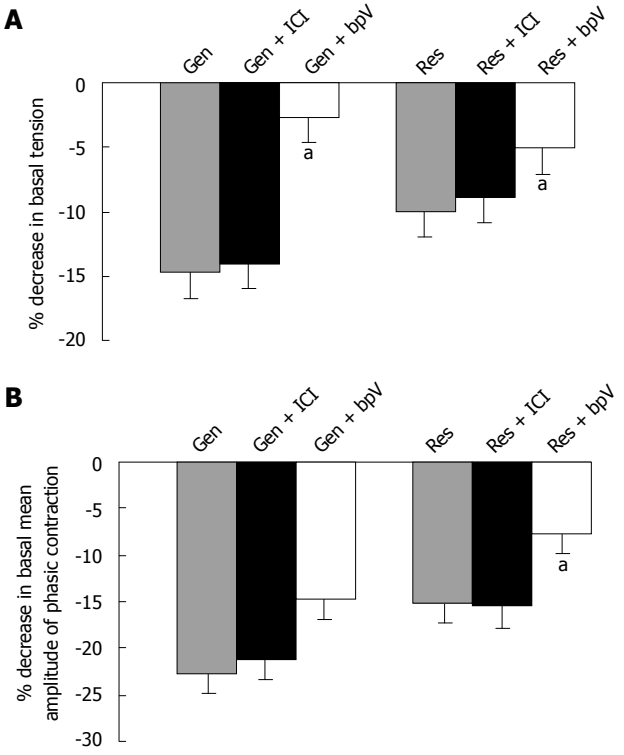

Figure 3 Effects of genistein (Gen, 10 μmol/L) and resveratrol (Res, 10 μmol/L) on the basal tension (**A**) and mean amplitude (**B**) of phasic contraction in isolated guinea pig gallbladder muscle strips after preincubation with ICI 182780 (ICI) or bpV (phen) (bpV) (n = 5). $^aP < 0.05$ *vs* corresponding Gen or Res group.

Effects of genistein and resveratrol on basal activities of gallbladder in the presence of ICI 182780 and bpV (phen)

The inhibitory effects induced by genistein and resveratrol in gallbladder muscle strips had no obvious change in the presence of the specific estrogen receptor inhibitor ICI 182780 (10 μmol/L) (Figure 3), but after incubating the strips with the potent protein tyrosine phosphatase inhibitor bpV (phen) (1 μmol/L), the inhibitory effects induced by genistein and resveratrol markedly attenuated (Figure 3). ICI 182780 (10 μmol/L) and bpV (phen) (1 μmol/L) alone had no obvious effect on basal activity.

Effects of genistein and resveratrol on biphasic contraction induced by ACh and $CaCl_2$

In calcium-free (0.01 mmol/L EGTA) Kreb's solution,

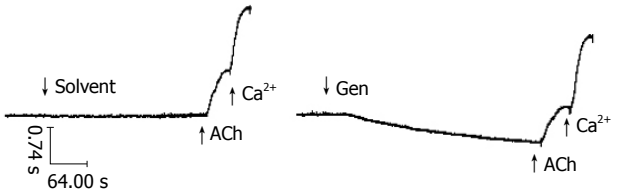

Figure 4 Traces of ACh and $CaCl_2$-induced contraction of gallbladder muscle strip in Ca^{2+}-free Kreb's solution in the absence and presence of genistein (Gen, 20 μmol/L).

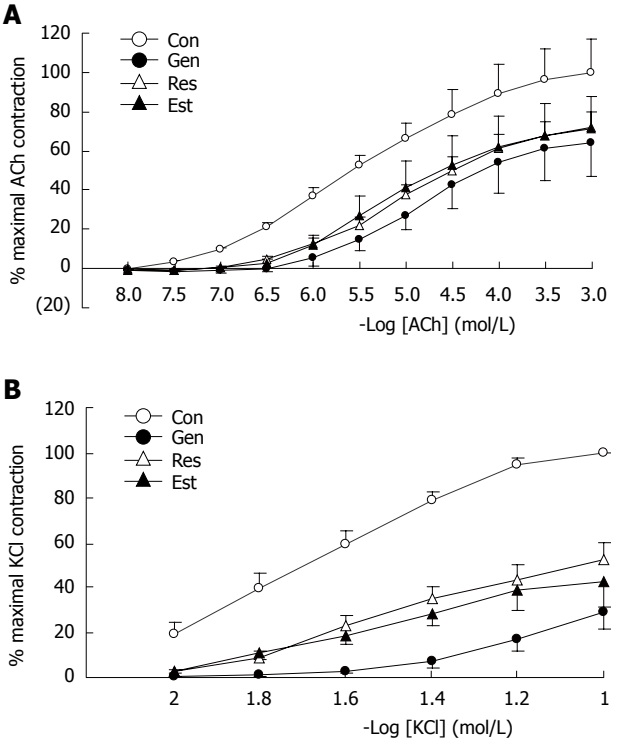

Figure 5 **A**: Line plots showing effects of genistein (Gen, 40 μmol/L), resveratrol (Res, 40 μmol/L) and 17β-estradiol (Est, 40 μmol/L) on ACh concentration-dependent contraction curves in isolated guinea pig gallbladder muscle strips (n = 6-7); **B**: Effects of genistein (Gen, 40 μmol/L), resveratrol (Res, 40 μmol/L) and 17β-estradiol (Est, 40 μmol/L) on KCl concentration-dependent contraction curves in isolated guinea pig gallbladder muscle strips (n = 12).

no spontaneous phasic contractions were observed, but ACh (10 μmol/L) could cause a transient contraction with the tensive increase of 0.89 ± 0.10 g. As soon as such contraction reached a plateau, $CaCl_2$ 10 mmol/L was rapidly added into the bath and another higher contractile response occurred with the tensive increase of 1.10 ± 0.18 g (n = 4). Genistein (20 μmol/L; Figure 4) and resveratrol (20 μmol/L) reduced the first contraction induced by ACh from 0.89 ± 0.10 g to 0.50 ± 0.18 g and 0.64 ± 0.15 g respectively (all $P < 0.05$, n = 4), but did not change the second contraction caused by $CaCl_2$ (1.23 ± 0.25 in genistein groups and 1.18 ± 0.15 in resveratrol groups *vs* 1.10 ± 0.18 g in control groups respectively, all $P > 0.05$, n = 4) in Ca^{2+}-free Kreb's solution.

Effects of genistein, resveratrol and 17β-estradiol on agonist-induced contractions

ACh (10^{-8}-10^{-3} mol/L) and KCl (10-100 mmol/L) elicited concentration-dependent contractile responses in isolated gallbladder muscle strips. However, genistein, resveratrol and 17β-estradiol significantly reduced the responses to ACh and KCl, and made their concentration-dependent contraction curves shift to the right (Figure 5). The pD_2 values of ACh in control and after incubation with 40 μmol/L genistein, 40 μmol/L resveratrol and 40 μmol/L 17β-estradiol were 3.97 ± 0.16, 3.38 ± 0.17 ($P < 0.05$ *vs* control, n = 6), 3.54 ± 0.08 ($P < 0.05$ *vs* control, n = 10) and 3.45 ± 0.14 ($P < 0.05$ *vs* control, n = 7), respectively. The pD_2 values of KCl in control and after incubation with 40 μmol/L genistein, 40 μmol/L resveratrol and 40 μmol/L 17β-estradiol were 1.61 ± 0.30, 0.70 ± 0.07 ($P < 0.05$ *vs* control, n = 11), 1.12 ± 0.03 ($P < 0.05$ *vs* control, n = 14) and 1.10 ± 0.05 ($P < 0.05$ *vs* control, n = 7).

DISCUSSION

The gallbladder and gut should be viewed as hormonally responsive organs. The normal physiology of which may be altered by the sex hormones[1]. Also, it is well established that cholelithiasis is more frequent in women than in men. This difference is usually explained by the effects of estrogens and progesterone on the metabolism of bile acids, biliary cholesterol secretion and saturation, and gallbladder motility[3,4]. As we know, gallbladder motility has an important role in the regulation of bile flow, its function disturbances may prevent normal bile flow and thus enhance the probability of common bile duct stone formation. Sex steroid hormone have inhibitory effects on the contractility which may be mediated by the inhibition of the mobilization of intracellular calcium and calcium influx in gallbladder smooth muscles[4].

Papers have shown that there are structural similarities between the steroidal nucleus of 17β-estradiol and the rigid ring structure of phytoestrogen genistein, and because both of them are lipid-soluble compounds and their molecular weight are not large, they can easily enter cytoplasm through the cellular membrane to affect expression of some genes. The affinity of genistein to the classic estrogen α receptor (ER_α) presented on reproductive organs is less than that of estrogen[17], but it has a similar affinity as estrogen for the novel estrogen β receptor (ER_β) in the vasculature[18]. As well as evidence that resveratrol exhibits variable degrees of estrogen receptor agonism in different test systems, and the similarity in structure between resveratrol and the synthetic estrogen diethylstilbestrol (DES; 4,4'-dihydroxy-trans-α, β-diethylstilbene) prompted us to investigate whether resveratrol might exhibit estrogenic activity in gallbladder motility[19]. The present study has shown that the phytoestrogen genistein and resveratrol can induce significant inhibitory effects on isolated gallbladder contractility in a similar way as 17β-estradiol does, and the effects were dose-dependent. Gallbladder smooth muscle cells have been shown to express functional ERs[2], so the effects of phytoestrogen genistein and resveratrol may be attributed to their combination with ERs, but our study demonstrates

that the inhibitory effects induced by genistein and resveratrol are unlikely to be mediated through the ER, as the actions of genistein and resveratrol had no obvious change in the presence of the pure and specific ER atagonist ICI 182780, although it can block not only the classical ER_α but also the novel ER_β[20]. These results suggest that the acute inhibitory effects caused by genistein, resveratrol and 17-β estradiol are not mediated by the classical estrogen receptor and are independent of gene-mediated events.

It is well known that genistein and resveratrol are tyrosine kinase inhibitors[12,13] and tyrosine kinase activity has been demonstrated to play a role in smooth muscle contractility[4,21]. In the present experiment, a potent protein tyrosine phosphatase inhibitor bpV (phen), which can prevent the decrease of protein tyrosine phosphorylation, markedly attenuated the inhibitory effects of 10 μmol/L genistein and 10 μmol/L resveratrol on gallbladder smooth muscle contractile activities. Our results suggest that tyrosine kinase inhibition is probably responsible for the inhibitory effects induced by genistein and resveratrol in gallbladder smooth muscle contractility. These results are supported by the evidences that tyrosine kinase inhibition contributes to the decrease of Ca^{2+} influx and Ca^{2+} mobilization[21,22].

The presence of cholinergic M receptors in guinea pig gallbladder smooth muscle cells has been reported[23]. ACh can activate receptor-operated calcium channels (ROCs) in the cellular membrane of gallbladder smooth muscle and increase calcium influx, while also activating G proteins and phospholipase C to produce inositol trisphosphate (IP_3) which causes calcium release from endoplasmic reticulum[4,23]. As we know, the contractile response to ACh comprises two distinct components in Ca^{2+}-free medium: an initial phasic component that results from IP_3-mediated release of Ca^{2+} from intracellular Ca^{2+} stores followed by a tonic component that requires addition of Ca^{2+} in the continuous presence of ACh, due to Ca^{2+} influx. This is so-called biphasic contraction induced by ACh and Ca^{2+}. In calcium-free Kreb's solution, genistein and resveratrol could significantly decrease ACh-induced contraction but they did not affect the latter $CaCl_2$-induced contraction. In normal Kreb's solution, genistein, resveratrol and 17β-estradiol could also reduce the contractile responses of ACh and shift their cumulative concentration-response curves rightward in a parallel manner. Considering these observations, it seems reasonable to suggest that the inhibition of Ca^{2+} release may involve in the inhibitory effects of genistein and resveratrol on gallbladder smooth muscle contractility.

Potential dependent calcium channels (PDCs) are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased, and it has been reported that potassium-stimulated gallbladder contraction depends exclusively upon the influx of extracellular calcium[4]. In the present experiment, genistein, resveratrol or 17β-estradiol could shift the KCl concentration-dependent contraction curves to the right in normal Kreb's solution and inhibited KCl concentration-dependent contractile responses in a noncompetitive manner. The results suggest that genistein and resveratrol may have Ca^{2+} antagonistic properties which are consistent with the effect of 17β-estradiol, and inhibit extracellular Ca^{2+} influx through PDC.

In summary, similar to 17β-estradiol, genistein and resveratrol have been shown to have a direct inhibitory effect on both the basal and agonist-stimulated contractile activity of guinea pig gallbladder *in vitro*.

COMMENTS

Background

Phytoestrogen genistein and resveratrol are structurally and functionally similar to estrogen and possess many physiological and pharmacological effects. Data indicate that genistein, resveratrol can inhibit vasocontractile responses and relax vascular smooth muscles by a Ca^{2+} antagonistic property which is similar to estradiol, but little is known about the effect of genistein and resveratrol on gallbladder smooth muscle motility.

Research frontiers

Gallbladder disease is more prevalent in women than men, and estrogen therapy has been associated with an increased incidence of gallbladder disease in both sexes, suggesting that hormones may play an important role in these conditions. Phytoestrogens such as genistein and resveratrol have estrogen agonistic/antagonistic effects, and are proposed to have potential as natural substitutes of estrogen therapy.

Innovations and breakthroughs

This study aim is to compare the direct effects of genistein and resveratrol on isolated gallbladder smooth muscle motility with that of 17β-estradiol both at rest and in response to stimulation, and to elucidate the underlying mechanisms.

Applications

The present results reflect the pharmacological actions of genistein and resveratrol and can provide the pharmacological guidance for the application of these compounds which are very valuable for medicine and nutrition.

Peer review

This is an interesting report of effects of genistein and resveratol on gallbladder contractility.

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