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PHYSIOLOGICAL STUDY OF THE EFFECTS OF GINGER OIL ON RAT UTERINE CONTRACTION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

Suranaree University of Technology

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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GINGER OIL/ZINGIBERACEAE/UTERUS/CITRAL/CAMPHENE

Ginger rhizomes (*Zingiber officinale* Roscoe) have been extensively studied for their pharmacological activities, but not for their physiological activities in smooth muscle. The aim of this study was to elucidate effects of ginger oil and its pure compounds (citral and camphene) on uterine contraction and investigate the mechanisms for the best position to exert the effects. Particular, the experiments were designed to determine whether the mechanisms depend on cyclooxygenase pathways, Ca^{2+}-CaM MLCK pathways, or non- Ca^{2+}-CaM MLCK pathways. The effects of ginger oil and its pure compounds on myometrial morphological features and the inflammatory process were also examined. Ginger oil was analyzed by GC-MS and dissolved in hexane (< 0.15%). The rats were humanely killed by cervical dislocations and the myometrial tissues dissected. The strips were immediately immersed into Krebs' solution containing in the organ bath and measured by PowerLab. The results showed that IC_{50} of ginger oil, citral, and camphene were 50 µl/100 ml, 2.2 mM, and 7.5 mM, respectively. They inhibited spontaneous and PGs-induced myometrial contraction. This is probably due to the inhibition of L-type Ca^{2+} channels. The effects of ginger oil and its pure compounds were reversible upon elevation of external Ca^{2+} concentration (from 2 to 5 mM). Without external Ca^{2+}, PGs elicited a small force that was inhibited by ginger oil, citral, and camphene. The myometrial contraction may be inhibited via inhibition of Ca^{2+}-CaM MLCK pathways. In addition, in the absence of external Ca^{2+}, they can inhibit force, presumably via inhibition of

non- Ca^{2+}-CaM MLCK pathways. Whereas, AA-induced contraction was decreased by ginger oil and its pure compounds. This inhibition (of force) may be exerted via inhibition of PKC or ROK pathway. PGs synthesis was inactive by using ginger oil and its pure compounds with DMSO, but they showed the contribution with indomethacin inhibiting COX-2 process. Consequently, they blockaded L-type Ca^{2+} Channels. These effects on myometrial contraction did not mediate by cAMP as the content is lower than 10^{-6} M. The normal feature of uterus in the presence of ginger oil and its pure compounds was detected by LM and TEM.

School of Biology

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Student's Signature 

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นพรัตน์ พุทธกาล : การศึกษาทางสรีรวิทยาถึงผลของน้ำมันขิงต่อการหดตัวของมดลูกหนู (PHYSIOLOGICAL STUDY OF THE EFFECTS OF GINGER OIL ON RAT UTERINE CONTRACTION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 182 หน้า.

ขิง (*Zingiber officinale* Roscoe) เป็นเครื่องเทศที่นิยมใช้ในการประกอบอาหาร และนำมาใช้เป็นสมุนไพรในการบรรเทาอาการปวดกล้ามเนื้อ และอาการอักเสบที่เกิดจากอิทธิพลของฮอร์โมนพรอสตาแกลนดินส์ ในการศึกษาวิจัยครั้งนี้ศึกษาถึงตำแหน่งที่น้ำมันขิงและสารมาตรฐาน (ซิทอล และ แคมฟีน) ออกฤทธิ์ยับยั้งการหดตัวของมดลูกหนู ในสภาวะที่ได้รับและไม่ได้รับการเหนี่ยวนำการหดตัวด้วยฮอร์โมนพรอสตาแกลนดินส์ ทำการทดลองโดยเน้นวิถีของ cyclooxygenase pathways, Ca^{2+}-CaM-MLCK pathways หรือ non- Ca^{2+}-CaM-MLCK pathways และตรวจพิจารณาลักษณะทางสัญฐานของกล้ามเนื้อมดลูกหนู โดยกลั่นน้ำขิงเพื่อนำไปวิเคราะห์สารประกอบด้วยเครื่อง GC-MS และนำมาทำละลายด้วยสารเฮกเซน (0.15%) จากนั้นทำให้หนูตายด้วยวิธีการเคลื่อนกระดูกคอ ทำการผ่าตัดเอามดลูกหนูมาแช่ในสารละลายเครบส์ที่บรรจุในอุปกรณ์ทดสอบการหดตัวของเนื้อเยื่อ ผลการศึกษาพบว่า ความเข้มข้นของน้ำมันขิงที่ 50 มล/100 มล ซิทอล 2.2 mM และแคมฟีน 7.5 mM สามารถยับยั้งการหดตัวของมดลูกหนูในสภาวะที่มีการเหนี่ยวนำการหดตัวทั้งที่ได้รับและไม่ได้รับฮอร์โมนพรอสตาแกลนดินส์ได้ 50% เมื่อมีการเพิ่มปริมาณแคลเซียมนอกเซลล์จาก 2 mM เป็น 5 mM ทำให้ฤทธิ์ในการยับยั้งการหดตัวของน้ำมันขิงและสารมาตรฐานกลับสู่การหดตัวเป็นปกติ นอกจากนี้น้ำมันขิงและสารมาตรฐานยังสามารถยับยั้งการหดตัวของมดลูกหนูที่ถูกเหนี่ยวนำด้วยกรดอะราชิโดนิกได้ โดยการยับยั้งดังกล่าวเกิดจากการปิดประตูแคลเซียม ในวิถีของ Ca^{2+}-CaM-MLCK pathways ขณะเดียวกันในการทดลอง ในภาวะที่ปราศจากแคลเซียมนอกเซลล์ (0-Ca) พบว่าน้ำมันขิงและสารมาตรฐานมีฤทธิ์ยับยั้งการหดตัวโดยเกี่ยวข้องกับวิถีของ non-Ca^{2+}-CaM-MLCK pathways โดยไม่ทำลายเนื้อเยื่อมดลูก จากการตรวจสอบด้วยกล้องจุลทรรศน์เลนส์ประกอบ กล้องจุลทรรศน์อิเล็กตรอน และทดสอบกับสาร BSA (5%) ส่วนน้ำมันขิงและสารมาตรฐานที่ทำละลายใน DMSO นั้นไม่มีผลต่อวิถีของ cyclooxygenase pathways แต่สามารถออกฤทธิ์ร่วมกับอินโดเมทาซินในการยับยั้งการหดตัวของมดลูกหนู และเป็นการยับยั้งการหดตัวที่ไม่เกี่ยวข้องกับวิถีของ cAMP pathways

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา _____

CHAPTER I

INTRODUCTION

1.1 Uterus and Its Functions

The **uterus** or **womb** is a major female reproductive organ of most mammals, including humans. One end, the cervix, opens into the vagina; the other is connected on both sides to the fallopian tubes. In mammals, the four main forms in which it is found are: bipartite, as in cows; bicornuate, as in pigs; simplex, as with the pear-shaped one found in humans and horses; and duplex, found in rodents.

The uterus is located in the pelvis immediately dorsal to the urinary bladder and ventral to the rectum. It is held in place by several ligaments. Outside of pregnancy, its size is several centimeters in diameter.

The uterus mostly consists of muscle, known as myometrium. The lining of the uterine cavity is called the endometrium. In most mammals, including humans, the endometrium builds a lining periodically which, if no pregnancy occurs, is shed or reabsorbed. Shedding of the endometrial lining in humans is responsible for monthly menstrual bleeding, known colloquially as woman's "period", throughout the fertile years of a female. In other mammals there may be cycles set as widely apart as six months or as frequently as a few days.

Although, there are morphological differences in the uteri among species, they have a unique function. The main function of the uterus is to accept fertilized ovum which (becomes) implanted into the endometrium, and derives nourishment

from blood vessels which develop exclusively for this purpose. The fertilized ovum becomes an embryo, which then develops into fetus and gestates until childbirth. In addition, the uterine changes during menstrual cycle are caused by changes in the plasma concentration levels of estrogen and progesterone. During the proliferative phase, an increasing plasma estrogen level stimulates growth of both the endomertium and the underlying uterine smooth muscle (myometrium) for their receptors. Then, following ovulation and formation of the corpus luteum (during the secretory phase), progesterone acts upon this estrogen primed endometrium to convert it to an actively secreting tissue. The changes are essential to make the endometrium a hospitable environment for implantation and nourishment of the developing embryo. Uterine quiescence is maintained by progesterone throughout pregnancy and is essential to prevent premature delivery.

1.1.1 Anatomy of the Uterus

The uterine wall is thick and composed of three layers. The endometrium is the inner mucosal layer lining the uterine cavity. It is covered with columnar epithelium and contains abundant tubular glands. The myometrium, a very thick, muscular layer, largely consists of bundles of smooth muscle fibers in longitudinal, circular, and spiral patterns and is interlaced with connective tissues. During the monthly female reproductive cycles and during pregnancy, the endometrium and myometrium extensively change. The perimetrium consists of an outer serosal layer, which covers the body of the uterus and part of the cervix (Shier et al., 2002).

1.1.2 Contractile Proteins

The structural and functional filaments of two major proteins in muscle cells are thick and thin (Broderick and Broderick, 1990) filaments. The former comprises thick myosin-containing filaments with thin actin-containing filaments. Their interaction governs the extent of smooth muscle contraction (Taggart and Morgan, 2007). It has been suggested that this contraction arises via Ca^{2+}-independent isoforms of PKC, possibly via uninhibited thin filaments (Horowitz et al., 1996).

The Thick Filaments

Myosin (thick filaments) consist of two heavy chains (MHC) that form a coiled rod-like structure together with a globular head domain, two regulatory light chains (MLC_{20}) and two essential light chains (MLC_{17}). One of each type of the light chain is associated with the head domain of MHC. Regulation of each myosin subunit, whether that be in terms of expression or post translational modification, may well participate in myometrial contractile adaptations with gestation. An elevation of Ca^{2+} results in the co-operative binding to the calcium binding canmodulin protein (CaM) and subsequent activation by Ca^{2+}-$(CaM)_4$ of the intracellular enzyme myosin light chain kinase (MLCK). Ca^{2+}-$(CaM)_4$-MLCK, in turn, acts to increase the serine/threonine phosphorylation of the regulatory light chains of myosin (MLC_{20}). A matching of the elevation of $[Ca^{2+}]_i$ to phosphorylation of MLC_{20} has been reported in uterine smooth muscle of many species and in response to diverse contractile stimuli (Taggart et al., 1997; Word et al., 1993; Word et al., 1994; Shojo and Kaneko, 2001)

The Thin Filaments

Contractile thin filaments consist mainly of an alpha helical coil of actin and associated proteins caldesmon and calponin (Marston and Redwood, 1991). Smooth muscle actin however, has been suggested to exist as part of both a contractile domain directly involved in force-generation events and a cytoskeletal domain important for structural integrity (Small and Gimona, 1991).

Calmodulin

Calmodulin was previously discussed in relation to the function of Ca^{2+} as a second messenger in hormone action. The calmodulin-Ca^{2+} complex thus formed combines with and activates MLCK, an enzyme that catalyzes the phosphorylation (addition of phosphate groups) of *MLC,* a component of the myosin cross bridges. In smooth muscle (unlike striated muscle), the phosphorylation of myosin cross bridges is the regulatory event that permits them to bind to actin and thereby produce a contraction. Calmodulin can bind four Ca^{2+} ions but may have two already bound at the c-terminal binding sites under resting conditions ie, low $[Ca^{2+}]_i$ (Bavley et al., 1996; Iohnson et al., 1996). Indeed, recently suggested a novel scheme where by a portion of calmodulin is tightly bound to the myofilaments and the Ca^{2+} (Keirse, 1995; Larcombe-McDouall et al., 1999) for contraction diffuses to this site. The Ca^{2+}-calmodulin (CaM) interaction introduces significant delay between $[Ca^{2+}]_i$ increase and ensuring an increase of force. The activation of MLCK after Ca^{2+}-calmodulin binding is also relatively slow and could be one of the rate-limiting steps in

contraction along with recruitment and diffusion of calmodulin (Somlyo and Somlyo, 1990).

Myosin Light Chain Kinase

Myosin light chain kinase (MLCK) of smooth muscle consists of an actin-binding domain at the N-terminal, the catalytic domain in the central portion, and the myosin-binding domain at C-terminal. The kinase activity is mediated by the catalytic domain that phosphorylates the myosin light-chain of 20 kDa (MLC_{20}), activating smooth muscle myosin to interact with actin (Nakamura et al., 2008).

Since MLCK is activated by Ca^{2+}-calmodulin complex, it is somewhat difficult to estimate the relative contribution of thick and thin filaments in the activation process of smooth muscle contraction. However, the affinity of caldesmon (CD) or calponin (CP) for Ca^{2+}-CaM complex is two to three orders of magnitude lower than that of Ca^{2+}-CaM complex for MLCK (Walsh, 1994). This finding suggests that Ca^{2+}-CaM activated MLCK is the main mechanism of the contraction that CD and CP act as second regulatory mechanisms (Savineau and Marthan, 1997). MLCK can be phosphorylated *in vitro* by several kinases including PKA, PKC and CaM kinase II (Nishikawa et al., 1978; Adelstein et al., 1978; Hashimoto and Hodering, 1990; Ikebe and Reardon, 1990; Stull et al., 1993). It has been presented that stimulation by agonists elevate the intracellular Ca^{2+} concentration of smooth muscle, causing Ca^{2+} to bind with calmodulin (CaM). CaM in conjunction with Ca^{2+} (Ca-CaM) activates MLCK. Myosin, thus phosphorylated at MLC_{20} by MLCK, is in an active form and interacts with actin to induce contraction (Bárány, 1979).

Myosin Light Chain Phosphatase

Myosin light chain (MLC) Phosphatase is physiologically responsible for the dephosphorylation of the MLC_{20}. The kinase is found to be bound tightly with myosin and is not dissociated from myosin under physiological ionic conditions, suggesting that under physiological condition the kinase is targeted for its substrate (Horowitz et al., 1996).

Force regulation in smooth muscle is dependent on the activities of MLC kinase and MLC phosphatase (Hartshorne et al., 1998; Gong et al., 1992). The activity of MLC kinase is regulated by Ca^{2+}-CaM (Hartshorne et al., 1998), whereas MLC phosphatase was originally thought to be constitutively active and unregulated (Hartshorne et al.,1998). However, there is abundant evidence that the activity of MLC phosphatase can be both inhibited to produce Ca^{2+} sensitization (Hartshorne et al., 1998; Somlyo and Somlyo, 1994; 1999) or an increase in force at a constant [Ca^{2+}]. Nitric oxide (NO) is the classical agent to produce Ca^{2+}desensitization by activating the soluble pool of guanylate cyclase, which in turn produces cGMP and leads to the activation of type I cGMP-dependent protein kinase (PKGI). PKGI mediates smooth muscle cell relaxation by several mechanisms. It has been demonstrated that PKGI acts on the K^+ channel to produce hyperpolarization of the smooth muscle, decreases Ca^{2+} flux, and also activates MLC phosphatase (Surks et al., 1999; Etter et al., 2001) to decrease the level of MLC_{20} phosphorylation and to produce smooth muscle relaxation.

1.1.3 Uterine Contractile Activity

Excitation-Contraction Coupling

Excitation-contraction (EC) coupling in smooth muscles is triggered by a sharp rise in the Ca^{2+} concentration within the cytoplasm of the muscle cells (Fox, 2004). The EC coupling starts with a depolarization of the plasma membrane that is the activation threshold of voltage-activated dihydropyridine-sensitive L-type Ca^{2+} channels and causes them to open. The opened Ca^{2+} channels allow the influx of Ca^{2+} that not only contributes to the further explosive depolarization of the plasmalemma, but also binds to the Ca^{2+} binding protein calmodulin (Matthew et al., 2004). Which is structurally similar to troponin in striated muscles, calmodulin was previously discussed in relation to the function of Ca^{2+} as a second messenger in hormone action. The Ca^{2+}-calmodulin complex thus formed combines with and activates MLCK, an enzyme that catalyzes the phosphorylation of MLC, a component of the myosin cross bridges (Fox, 2004) and thereby produce a contraction.

Uterine Smooth Muscle Contraction

Muscle contraction is turned on when sufficient amounts of Ca^{2+} bind to troponin protein. This occurs when the concentration of Ca^{2+} of the sarcoplasm rises above 10^{-6} molar (Fox, 2004). During normal contraction, when the cross bridges attach to actin, they undergo power strokes and cause muscle contraction. The contractile state of smooth muscle is determined predominantly by the level of phosphorylated myosin, achieved largely via MLCK whose activity is regulated by calmodulin (Somlyo and Somlyo, 1994; Walsh et al., 1996). In order for muscle to relax, therefore, the attachment of myosin cross bridges to actin must be prevented. The regulation of cross-bridge attachment to actin is a function of two proteins that are associated with actin in the thin filaments.

Uterine Smooth Muscle Relaxation

The muscle relaxation is produced by the active transport of Ca^{2+} out of the sarcoplasmic reticulum (SR). The SR is a modified endoplasmic reticulum, consisting of interconnected sacs and tubes that surround each myofibril within the muscle cell. Relaxation of the smooth muscle follows the closing of the Ca^{2+} channels and lowering of the cytoplasmic Ca^{2+} concentration by the action of Ca^{2+}-ATPase active transport pumps (Fox, 2004). Under those conditions, calmodulin dissociates from the myosin light-chain kinase, thereby inactivating this enzyme. The phosphate groups that were added to the myosin are then removed by a different enzyme, a myosin phosphatase. Dephosphorylation inhibits the cross bridge from binding to actin and producing another power stroke.

Although the uterus is myogenic, myometrial activity can be regulated by both adrenergic and cholinergic nerves. Adrenergic stimulation caused both contraction and relaxation of the uterine smooth muscle through smooth muscle α-(excitatory) and β- (inhibitory) adrenoreceptors (Marshall, 1970; O'Donnell et al., 1978; Digges, 1982). On the other hand chlorinergic stimulation has been shown to cause the contraction of the myometrial through muscarinic receptors present on the smooth muscle cells (Nakanishi and Wood, 1971; Hollingsworth, 1975; Morizaki et al., 1989). Neuronal modulation, especially the β-adrenergic agonists (β-mimertics) are the most commonly used tocolytic agents for the prevention of pre-term delivery (Monga and Creasy, 1995). The rationale for using these compounds is based on their

ability to increase adenosine 3́, 5́ -cyclic monophosphate (cAMP) or guanosine 3́, 5́ -cyclic monophosphate (cGMP) level in smooth muscle of the uterus through binding to specific receptors linked to the stimulatory guanine neucleotide-dependent regulatory protein ($Gi_{(s)}$), which, in turn, leading to uterine relaxation. cAMP and cGMP are important second messengers that control many physiological processes, including smooth muscle relaxation (Diamond, 1978). Adenylyl cyclase and guanylyl cyclase synthesize cAMP and cGMP, respectively and phosphodiesterases (PDE) enzyme degrades them (Fig. 1.1). The proposed mechanism for action of cAMP and cGMP in smooth muscle relaxation can be described (see below).

Cyclic Nucleotide-Induced Relaxation

cAMP is an important intracellular second messenger in many tissues and mediates the effect of multiple drugs and hormones. It is known that cAMP produces relaxation of smooth muscle by activation of cAMP dependent protein kinase (PKA) which interferes with several processes involved in smooth muscle contraction (Wray, 1993). cAMP causes relaxation by lowering in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by: 1) increasing extrusion of Ca^{2+} due to stimulation of both Ca^{2+} transport and Ca^{2+} ATPase by the plasma membrane; 2) stimulating the Na^{+}-K^{+} pump, thereby lowering $[Na]_i$ which in turn enhance Ca^{2+} efflux on Na^{+}- Ca^{2+} exchange; 3) causing internal sequestration of Ca^{2+} (Casteels and Raeymaekers, 1979; Mueller and van Breemen, 1979); and 4) inhibiting Ca^{2+} influx, although this has not been demonstrated directly in the uterus (Scheid et al., 1979; Bullbring and den Hertog, 1980; Wray, 1993). However, recently data suggest that the relation between $[Ca^{2+}]_i$

and myosin light chain phosphorylaiton is variable and depends on the form of stimulation (Rembold, 1992). Although these different mechanisms have been proposed by respective researchers, an effort to compare and evaluate these four concepts in various smooth muscles had been neglected up to the present. The role of cAMP pathway in regulation of smooth muscle contraction is shown in Figure 1.1

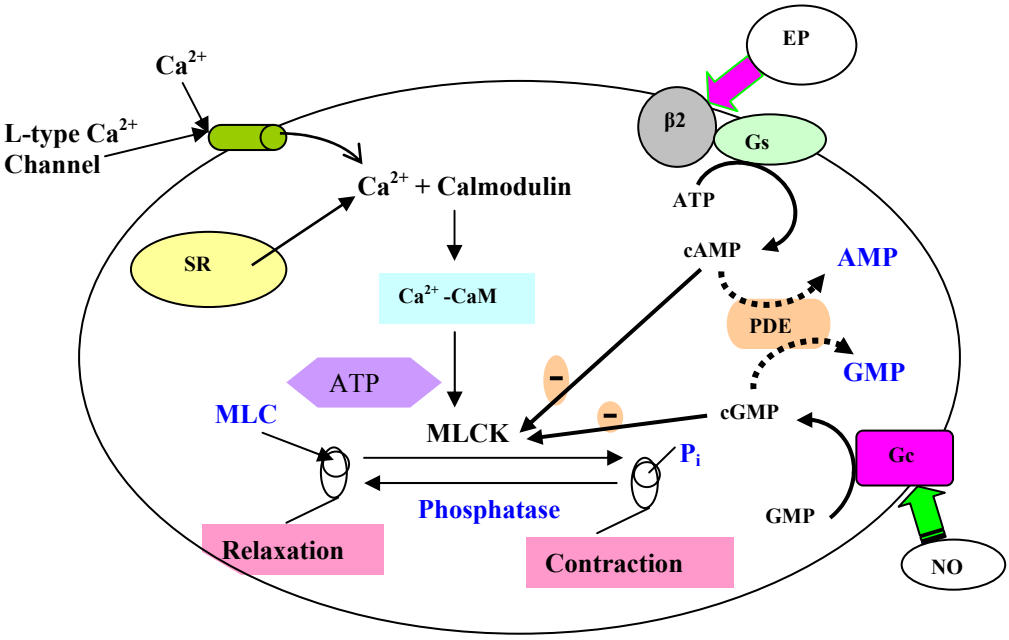

Figure 1.1 Schematic representation of the smooth muscle contraction.

Abbreviations: SR, sarcoplasmic retivulum; Gq, Gs-protein; MLC, myosin light chain; MLCK, myosin light chain kinase; Pi, phosphorylation myosin; β_2, beta-2- adrenergic receptor; cAMP, cyclic adenosine 3́, 5́ - monophosphate; cGMP, cyclic guanosine 3́, 5́ - monophosphate, NO; nitric oxide, G_c-protein; EP, epinephrine, and PDE, phospodiesterase.

During the last decade, numerous studies have demonstrated the modulation of inflammatory cell activations by selective PDE4 inhibitors. It is now established that an elevation of cAMP is able to inhibit some of inflammatory processes (Lagente et al., 2005). The increase of intracellular cAMP can be achieved through receptor activation or inhibition of cAMP breakdown (Conti et al., 1995). PDEs are responsible for the breakdown of intracellular cyclic nucleotides, from which PDE4 are the major cAMP metabolizing isoenzymes found in inflammatory and immune cells (Lagente et al., 2005). The first generation of PDE4 inhibitors, although potent anti-inflammatory agents, failed as pharmaceuticals owning to their emetic and gastric side-effects (Mackenzie, 2004). It should be noted that the inhibitory effect of cAMP was demonstrated under special conditions using high concentrations of cAMP ($\geq 10^{-4}$ M) in the presence of fluoride or theophylline or by the use of protein kinase. In contrast, the study reported that 3×10^{-6} M cAMP had no effect on 10^{-5} M Ca^{2+}-induced contraction of saponin-treated skinned smooth muscle in the presence of exogenous protein kinase (Itoh et al., 1982). According to Ruegg et al. (1983), the inhibitory effect of cAMP was best observed when a low concentration of Ca^{2+} was used for the contraction. Nevertheless, the present observation shows that 10^{-5} M cAMP had no effect on the contraction induced with 10^{-6} M Ca^{2+} or 10^{-5} M Ca^{2+} in the saponin-treated skinned preparation. The reason for this report is still unclear for the other smooth muscle. Since this problem is very important for understanding the Ca^{2+} regulation in smooth muscle, further studies are required.

It has been suggested that another nucleotide, cGMP, also act as an intracellular mediator for relaxation in some type of smooth muscles. Through phosphorylation of PKG, cGMP is thought to cause a decrease in cytosolic Ca^{2+} and Ca^{2+} sensitivity of contractile proteins. Another mechanism by which cGMP may promote relaxation of smooth muscle cells is membrane hyperpolarisation as a consequence of K^+ channel activation (Zhou et al., 2000). Thus, the role of cyclic nucleotide both cAMP and cGMP in uterine relaxation is supported by several lines of evidence. In addition, it has been proposed that the maintenance of uterine quiescence during pregnancy is stimulated by cAMP and cGMP (Lopez Bernal et al., 1995; Telfe et al., 2001).

1.2 Calcium Signaling and Uterine Contraction

1.2.1 L-type Ca^{2+} Channels

The properties of myometrial ion channels and their regulation by voltage and agonist-occupied receptors have been reviewed recently (Sanborn, 1995). Ca^{2+} channels are expressed in the myometrium, including L-type voltage sensitive channels. Of course, inward current by producing depolarization will also lead to the opening of L-type Ca^{2+}currents; hence there will be a synergy in their effects. T-type Ca^{2+} channels are also voltage sensitive but open at more negative potentials than L-type Ca^{2+} channels (-60 mV compared with -40 mV, respectively), have a smaller conductance, and have peak currents at around -30 mV compared +10 mV for L-type channels in the uterine cell (Triggle, 1998). The L-type Ca^{2+} channel opening is the main source of the Ca^{2+} which activates the myofilaments, but as mentioned above, IP_3 induced Ca^{2+} release may make a contribution via release of Ca^{2+} to the deep

cytoplasm. It is also the case that the relation between force and Ca^{2+} may be altered, a process known as Ca^{2+} (de) sensitization. One of the main modulatory pathways is agonists, via Rho associated kinase (Wray et al., 2003; Somlyo and Somlyo, 2003; Gerthoffer, 2005) and other agents, altering the activity of the MLCP. Recent data has shown that the Rho kinase pathway can be activated in the absence of agonist, e.g. by the action potential or high-K^+ depolarization (Shabir et al., 2004; Ratz et al., 2005).

1.2.2 Calcium from the SR

Reticular pattern of the SR of the uterine myocytes is an interconnecting membrane system of tubules and cisternae found throughout the cytoplasm. The SR is able to take up Ca^{2+}agonist the electrochemical gradient due to ATP-dependent Ca^{2+} pump in the SR membrane (Shmygol and Wray, 2004). The role of the SR is to feedback and limit contractility by contribution of Ca^{2+} induced Ca^{2+} release (CICR) through ryanodine (RyR) gated calcium channels producing force (Taggart and Wray, 1998). This may act to limit contractions and act as a calcium sink, rather than to amplify contractility (Kupittayanant et al., 2002). As mentioned above, a rise in intracellular [Ca^{2+}] is associated with contraction. The rise of $[Ca^{2+}]_i$ for myometrial contraction may come from two sources, an extraclellular Ca^{2+} entry and Ca^{2+}-released from the SR store. There are two types of Ca^{2+} release channels in the SR membrane: those gated by Ca^{2+} and known as ryanodine receptors (RyR) and those gated by IP_3, IP_3 recepter (IP_3R). Their expression is species dependent (Byrdyga et al., 1995). The Ca^{2+}-released from the store can occur through inositol trisphosphate (IP_3) gated channels giving rise to IICR via IP_3R on the SR membrane, while the RyR channels are also physiologically activated by Ca^{2+} itself, giving rise to CICR. The IP_3

is generated when agonists such as prostaglandins (PGs) bind to their receptors on the membrane, causing IP_3-induced Ca^{2+} release (IICR) from the SR (Wray, 1993; Luckas et al., 1999).

Recent work has clearly shown that Ca^{2+} signaling and contractility are increased in myometrial preparation if SR Ca^{2+} release is inhibited (Taggart and Wray, 1998; Kupittayanant et al., 2002). However, the SR of smooth muscles is less developed than that of skeletal muscles, and Ca^{2+} release from this organelle may account for only the initial phase of smooth muscle contraction. In the mouse, rat and human myometrium pharmacological inhibition of the SR Ca^{2+} pump, e.g. by cyclopiazonic acid (CPA), causes depletion of Ca^{2+} from the SR and increase cytosolic [Ca^{2+}] and contraction (Taggart and Wray, 1998; Tribe et al., 2000).

1.2.3 Calcium Sensitization

An increase in cytoplasmic [Ca^{2+}] is the key event in excitation-contraction coupling in smooth muscle and the relationship linking the $[Ca^{2+}]_i$ value to force of contraction represents the Ca^{2+} sensitivity of the contractile apparatus (Savineau and Marthan, 1997). Recently, it has become evident that agonist-mediated Ca^{2+}-sensitisation has been observed in permeabilized myometrial preparations-where myofilament activating Ca^{2+} can be clamped at sub-maximal levels-of rat, guinea-pig and human (Izumi et al., 1994; Izumi et al., 1996; Somlyo and Somlyo, 1999; Lee et al., 2001; Williams et al., 2005). In recent years, the focus of molecular mechanisms mediating Ca^{2+}-sensitisation of smooth muscle contractility has centered around signaling pathways that impair myosin phosphatase activity, thereby elevating MLC_{20}

phosphorylation and force. Additionally, the similarity in the effects of inhibiting MLCK in human and rat uterus are in agreement with previous data, suggesting that the basic mechanism of contraction is the same in both species (Wray, 1993).

1.3 Pathophysiological Problems of the Uterus

Several pathological conditions of the uterus has been reported. These include dysmenorrhea or painful menstruation.

Dysmenorrhea

Dysmenorrhea is one of the most common gynecological conditions, and is a leading cause of absenteeism by women from work, school and other activities. It is estimated to affect almost half of all women at some time during their childbearing years, usually appearing during adolescence and tending to decrease with age and following pregnancy (Owen, 1984). It is characterized by pain occurring on the first day of menses, usually coinciding with the onset of flow, but may not be present until the second day. The term dysmenorrhea is derived from the Greek words *dys,* meaning difficult/painful/abnormal, *meno,* meaning month, and *rrhea,* meaning flow (Gerbie, 1987).

The symptom may vary among women. Lower abdominal cramping and pain that may radiate to the thighs and lower back is the most prevalent symptom (Cahill, 1986). Headache, nausea, constipation or diarrhea, and urinary frequency are often present, and vomiting may also occur (Owen, 1984; Gerbie, 1987). The symptoms tend to peak after 24 hours and usually subside after 2 days. While many women suffer mild discomfort during menstruation, dysmenorrhea is present if pain prevents

normal activity and requires over-the-counter or prescription medication (Gerbie, 1987).

There are three types of dysmenorrhea: primary, secondary, and membranous. *Primary dysmenorrhea* is characterized by the absence of an organic etiology, while *secondary dysmenorrhea* is associated with specific diseases or disorders. *Membranous dysmenorrhea* (uterine cast) is rare and causes intense cramping pain as a result of the passage of the intact endometrial cast through an undilated cervix (Gerbie, 1987). A majority of women suffering from dysmenorrhea are diagnosed with primary dysmenorrhea.

Primary dysmenorrhea is due to the production of PGs. PGs are hormone-like compounds that function as mediators of a variety of physiological responses such as *inflammation*, muscle contraction, vascular dilation, and platelet aggregation. They are modified forms of unsaturated fatty acids, via the cyclooxygenase pathway, that are synthesized in virtually all cells of the body (Lavin, 1986). Studies have demonstrated that varying PG levels in the female reproductive tract affect the cyclic regression of the corpus luteum and the shedding of the endometrium. PGs may also mediate the effect of luteinizing hormone on ovulation (Budoff, 1983). The association between the symptoms of dysmenorrheal and intrauterine production of PGs goes back 49 years to the report of Pickles (Pickles, 1957), who first identified a substance in menstrual fluid which stimulated contractions of human uterine smooth muscle strips. This menstrual stimulant was subsequently found to contain $PGF_{2\alpha}$ and PGE_2, with the PGF/PGE ratio higher in the endometrium and menstrual fluid of women with primary dysmenorrheal (Pickles, 1957). $PGF_{2\alpha}$ and PGE_2 have opposing vascular effects causing vasoconstriction and vasodilation, respectively (Rees et al.,

1984). While $PGF_{2\alpha}$ administration stimulates uterine contractility during all phases of the menstrual cycle, PGE_2 may inhibit myometrial contractility during menstruation and stimulate it during the proliferative and luteal phases (Rees et al., 1984). Since they are both formed from a common precursor, arachidonic acid (AA), the increase in $PGF_{2\alpha}/PGE_2$ ratio indicates that synthesis can be directed preferentially towards the PGF compounds (Downie et al., 1974). Several studies suggest that women with primary dysmenorrhea have elevated concentrations of $PGF_{2\alpha}$ and/or its metabolites in the endometrium, menstrual fluid, and peripheral circulation (Rees et al., 1984; Willman et al., 1976; Lundstrom and Green, 1978). These findings have led to the hypothesis that painful menstruation may be due to hypertonicity of the myometrium with accompanying uterine ischemia caused by the local release of excessive amounts of PGs (Lundstrom et al., 1978). Furthermore, escape of PGs from the uterus into the systemic circulation could be responsible for other symptoms of dysmenorrhea such as gastrointestinal disturbances, faintness, dizziness, and headaches. This theory is supported by several research findings: 1) higher PG levels (especially $PGF_{2\alpha}$) during the secretory phase than in the proliferative phase of the menstrual cycle (Downie et al., 1974; Willman et al., 1976; Singh et al., 1975; Levitt et al., 1975); 2) high PG levels and high $PGF_{2\alpha}/PGE_2$ ratio found in the endometrium and menstrual fluid of women with dysmenorrheal (Budoff, 1983; Rees et al., 1984; Willman et al., 1976; Lundstrom and Green, 1978); 3) administration which are produces symptoms similar to dysmenorrheal (Roth-Brandel et al., 1970); and 4) PG inhibitors successfully relieve symptoms of dysmenorrheal (Lunstrom and Green, 1978). The association between the symptoms of dysmenorrheal and intrauterine production of PGs is depicted in Fig. 1.2

Treatment methods of primary dysmenorrhea include medications for pain and oral contraceptive pills to regulate the menstrual cycle. Nutritional and lifestyle medications play an important role, as well. In addition, herbal therapies have a long history of use in the management of dysmenorrheal.

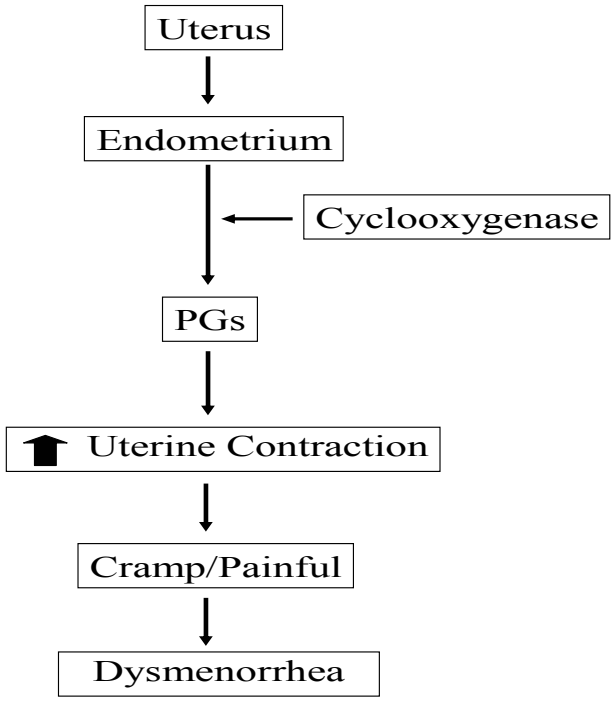

Figure 1.2 Schematic representation of the association between the symptoms of dysmenorrheal and intrauterine production of PGs.

PG synthetase inhibitors (non-steroidal anti-inflammatory drugs), such as ibuprofen, mefanamic acid, naproxen, and indomethacin, have been used as analgesic treatment for dysmenorrhea since the early 1970s (Owen, 1984). Prior to their discovery, women who had dysmenorrhea were dependent largely on narcotics or oral

contraceptives for pain relief (Budoff, 1983). PG inhibitors block PG synthesis early in the inflammatory reaction by inhibiting the cyclooxygenase pathway. Once pain has become severe, relief is unlikely. However, these drugs should not be used prior to the onset of menses because of their teratogenic potential (Gerbie, 1987). In a comprehensive review of clinical trials of PG inhibitors in the treatment of primary dysmenorrhea, it was found that significant pain relief was reported for each of the PG inhibitors for the majority of women (Owen, 1984). However, the authors concluded that 9% to 22% of dysmenorrheic women will not benefit from PG inhibitor treatment, possibly because some of these women may have secondary dysmenorrhea. While PG inhibitors are generally recognized as effective against pain, there are drawbacks. These drugs are not selective in their inhibition of PGs, translating to a reduction of all PGs, good or bad. In addition, possible side effects include dizziness, headache, nausea, vomiting, heartburn, and diarrhea, as well as gastrointestinal tract damage with protracted use (Cahill, 1986; Bjarnason et al., 1986).

Cyclic administration of oral contraceptives, usually in the lowest dosage but occasionally with increased estrogen, is also used to alleviate pain. The mechanism of pain relief may be related to absence of ovulation or to altered endometrium resulting in decreased prostaglandin production during the luteal phase (Gerbie, 1987; Budoff, 1983). Surgery is a rare form of intervention used in women who do not respond to medication.

1.4 Herbal Medication

Botanical medicines have been used to treat the symptoms of dysmenorrheal for centuries throughout the world (Shils et al., 1994; Bensky and Gamble, 1993). Herbs with a long history of use in treating women's problems include cramp bark *(Viburnum opulus)* and blue cohosh *(Caulophyllum thalictroides)*. These plants relax the uterine muscle by acting as antispasmodics and are used to relieve cramping, along with pain in the lower back and thighs (Brinker, 1997; Jarboe et al., 1966; Mabey, 1988); ginger root *(Zingiber officinale)*, an inhibitor of prostaglandin synthesis, has been used for thousands of years for its anti-inflammatory properties (Srivastava and Mustafa, 1989; Taymor et al., 1964); wild lettuce leaf *(Lactuca elongata)* has been used since ancient times for its pain-relieving and calmative effects (Weiner and Weiner, 1994), and black cohosh *(Cimicifuga racemosa)* has antispasmodic and analgesic properties, easing cramping and muscle tension (Mabey, 1988). Dong quai *(Angelica sinensis)* demonstrates uterine tonic activity, causing an initial increase in uterine contraction followed by relaxation (Ozaki and Ma, 1990). Growing evidence indicates that essential oils are useful for alleviation of dysmenorrheal sequalee (Ostad et al., 2001; Ostad et al., 2004).

As stated above, there are still far too may women who encounter dysmenorrheal difficulties. The cost of these, in terms of the socio-economic impact of dysmenorrhea, such as absenteeism from work and school, as well as disruption of social and athletic activities, are high, and fuel both clinical and scientific endeavors directed towards prevention and treatment. It is a need therefore to understand the underlying physiological mechanisms, and ultimately to improve prevention and treatment. The thesis proposal is directed to the study of hyperactivity of the uterus during $PGF_{2\alpha}$-and PGE_2-induced contraction (as a model of dysmenorrhea) and the

effects of selected herbs on it. I had been screening the physiological effects of plant oils extracted from some Thai medicinal plants on rat uterine relaxation. In the screening test, oils obtained from five plants including *Curcuma longa* Linn., *Curcuma zedoaria Rose., Boesenbergia pandurata* Roxb., *Ocinum bacilicum* Linn., *Ocinum sanctum* Linn., *Cymbopogon citraus* Stapf*., Zingiber cassumunar* Roxb., and *Zingiber officinale* Roscoe were used. Preliminary data showed that ginger oil extracted from *Zingiber officinale* Roscoe showed a relaxation effect of the uterus. Ginger oil is, therefore, will be used in this thesis.

Ginger Rhizomes (*Zingiber officinale* Roscoe*)*

Ginger rhizomes contain both volatile oils and nonvolatile pungent compounds which can be extracted with solvents such as acetone or alcohol including steam- and hydro-distillation. The hydro-distillation study showed that the volatile oil compounds of fresh ginger rhizomes consist mainly of monoterpenes, such as β-phellandrene 10.9%, camphene 11.85%, linalool 12.17%, geranial 2.2%, zingiberene 17.44%, β-sesquiphellandrene 5.01%, neral 4.17%, α-bisabolene 7.96%, α-curcumene 4.34%, α-farnesene 12.68% and α-muurolene 5.36% (Zhou et al., 2006). According to these investigations, major constituents in the essential oil of the ginger rhizomes, main constituents in the essential oil of the various kinds of the Japanese ginger rhizomes, including the young shoots, are monoterpenes (Sakamura and Hayashi, 1978; Sakamura, 1987). Previous studies indicated that the essential oil of the green ginger from Fiji has a high content of monoterpene aldehydes, such as neral and geranial (Smith and Rhobinson, 1981). Moreover, the steam distillation of Australian ginger rhizomes has been characterized by very high citral levels (51-71%) and

relatively low levels of the sesquiterpene hydrocarbons typical of ginger oil (Wohlmuth et al., 2006). The main flavour components of ginger rhizome are the monoterpene aldehydes geranial and neral, which impart a lemon-like quality to the essential oil, and pungent phenolic derivatives, the most important of which is 6-gingerol (Sakamula, 1987).

1.5 Aims

There are two main aims to the program of this work, which are interconnected: 1) to investigate the effects of ginger oil and some of its active compounds on prostaglandins-induced contraction, in particular their effects on uterine relaxation; and 2) to increase our understandings of the physiological mechanisms where by ginger oil inhibits uterine contractility arising either spontaneously or $PGF_{2\alpha}$ and PGE_2 stimulation. Due to some difficulties to obtain human myometrial tissues, rat myometrial tissues will be used in the study. However, it has been suggested that the mechanisms of uterine smooth muscle contraction found in rats are most likely the same as in humans (Wray et al., 2001).

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CHAPTER II

GENERAL MATERIALS AND METHODS

This chapter will give a general description of major materials and methods used in the work prensented in this thesis. More details pertinent to each study are given in each chapter.

2.1 Plant Preparations

2.1.1 Plant Collections

Mature plants of *Z. officinale* rhizomes were harvasted and purchased from the farmer at a local garden in Damnoen Saduak, Rachaburi, Thailand. The raw plant materials were subjected to appropriate preliminary processing, including elimination of undesirable materials and contaminants, washing (to move excess soil). Prior to processing, the plant materials approximately 40 kg were kept away from rain, moisture and other conditions. Plant materials were dried in the open air (shaded from direct sunlight) for the future procedure. The samples were classified and confirmed at the Royal Forest Department, Bangkok Thailand. The voucher specimen was kept in the laboratory for future references (Herbarium No. 42629).

Botanical and Morphological Description

Z. officinale, is known as "Ginger plant" or other names: Khing, Khing daeng (Chanthaburi), Khing phueak (Chaingmia), Sa-e (Karen-Mae Hong Son), Khing klaeng (Medicinal Plant Information Center, 1992).

An original name of the Sunskrit name 'Singabera' gave rise to Greek 'Zingiberi' and later the generic name, Zingiber. Zingiberaceae was a distinctive family in order Zingiberales and genus Zingiber (Heywood, 1993). Such *Z. officinale* is a monocotyledon, usually propagates as a herbaceous tropical perennial and origenates in South-East Asia, probably in India (Purseglove et al., 1981).

Ginger was obtained from the rhizomes of the plant *Z. oficinale* Roscoe. The rhizome was branched, a horizontal underground stem as root stock (Harris and Harris, 1954), fleshy rhizome (white or pale yellow) and frequently possess tuberous roots and also possess oil cells (Hardman, 1972). The aerial stem was invariably short about 2 to 4 feet long prerenial that produced grass-like leaves up to a foot long, and usually leafless, but sometimes quite leafy. Leaves were lanceolate (much longer than wide, with the widest point below the middle). The leaves emerge from the rhizomes as two distinct ranks and toward the base they consist of open or closed sheaths (Heywood, 1993).

Inflorescences were borne separately on a bladeless leaf-sheath; consisting of flowers zygomorphic, with bracts and bracteoles subtending the flowers, bracts closely appressed against each other; calyx shortly 3 lobed; corolla tubular, divided into 3 subequal lobes; fertile stamen one only; very rarely flowers. Fruit was a dehiscent capsule (Medicinal plant information center, 1992).

2.1.2 Extraction of the Ginger Oil

Fresh rhizomes of *Z. officinale* were separated from the arial stems and prepared using distillation technique. Rhizomes were washed and chopped into pieces approximately 1 x 3 x 7 mm. Approximately 40 kg of this material was hydrodistilled in a Cleveneaur's distillation apparatus (Fig. 2.1) at 140 to 200°C for 24 hr. After allowing the system to cool down, the volatile oil was yielded and stored at 4°C until used. The extracted oil was analyzed for the constituents by gas chromatography-mass spectrometry (GC-MS), it can be seen in Chapter IV. This part of work was conducted at the Chemical Research Institute, Rajamangala University of Technology Thanaburi, Pathumtani, Thailand.

Figure 2.1 A Cleveneaur's distillation apparatus used in the extraction process.

2.1.3 Making Stock Solution

A stock solution was obtained by dissolving small aliquots of the oil in hexane (1:1 v/v). The stock solution was further diluted using Krebs' solution to attain the final concentration. The final concentration of hexane in any dilution was less than 0.15%.

2.2 Animal Preparations

2.2.1 Housing

Rats were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, SUT.

Twenty five non-pregnant Wistar rats (200–250g) were housed in the animal house of the SUT under a controlled environment (23–25 ^{o}C) and illumination (12 hr light, 12 hr dark) room. Rats were given tap water and a standard diet ad libitum.

2.2.2 Myometrial Tissue Preparations

The rats were humanely killed by cervical dislocation. Myometrial tissues were obtained. The uterus was removed and immediately immersed in buffered physiological Krebs' solution (pH 7.40) containing (mM): 154 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 12 glucose; 2 $CaCl_2$, and 10 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]] [HEPES]. The uterus was then placed in a shallow dissecting dish containing Krebs' solution at room temperature under a microscope. The longitudinal layer was separated from the endometrium and circular layers. Five or six

strips (1-2 mm x 0.5 mm x 10 mm) of longitudinal fibres were then dissected. The strips were either used immediately or stored for a maximum of 12 hr at 4 ^{o}C.

2.2.3 Histological Tissue Preparations

The strips were immersed in buffer physiological Krebs' solution as a control, in Krebs' solution containing ginger oil, or ginger oil related compounds for 30 min. The strips were fixed in 10% formalin until analyzed for histological features.

2.2.4 Myometrial Tissue Staining Preparations for LM and TEM

2.2.4.1 LM Staining Preparations

The horns of rat uteri were cut into short segmenta. These strips were dehydrated in an ascending ethanol series (75%, 85%, 95%, 100% and 100%, 1 hr each) and then removed the sections into pure xylene for 2 min. The sections were embedded with xylene : paraplast (3 : 1 and 1 : 1, 15 min each) with pure paraplast for 1 hr. Post-embedded tissues were cut approximately 5 μm with microtome and moved into water bath at 60°C for 10 min. The tissues were mounted on slides in the slide warmer at 60°C and then twice immersed the slides in pure xylene (5 and 2 min, respectively). Next, tissues were hydrated in a descending ethanol series (100%, 95%, 70%, 50%, 30%, distill water, 2 min each) and stained with heamatoxylin according to standard protocols. All tissues were dehydrated in a descending ethanol series (35%, 50%, 70%, 95%, 2 min each) and stained with eosin for 1 min. Finally, the slides were immersed in 95% ethanol (2-3 min) and 100% ethanol (1 min), pure xylene (5 min) and then covered with cover slip after xylene clearing for LM study.

2.2.4.2 TEM Staining Preparations

The horns of rat uteri were cut into short segmenta. These strips were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 overnight at 4°C then washed in the same buffer 3 times. Post-fixed with 1% osmium tetroxide for 2 hr then rinsed in distill water 3 times. Gold coated uterus on stubs were examined for TEM, after post-fixing the samples were dehydrated in graded acetone at concentrations of 20%, 40%, 60%, 80%, 100% and 100%, then infiltrated and embedded in Spur's resin. Upon identifying suitable areas from the semithin sections, ultrathin sections (<100 nm thick) of mainly transverse and longitudinal orientation to the uterine tissues were cut with diamond knife, mounted on copper grids then stained with lead citrate and uranyl acetate. The ultrathin sections on copper grids were examined using TEM (JEM 1230) at Central Instrumentation Unit, Faculty of Science, Mahasarakham University (Phungnoi and Narkkong, 2007).

2.3 Measurements of Tension

The uterine strip was mounted vertically under resting tension of 1g in a single chamber (25 ml) tissue bath connected to a force transducer (see Fig. 2.2).

The organ bath contained Krebs' solution maintained at pH 7.4, temperature of 37 ^{o}C, and gassed with carbogen (95% O_2 and 5% CO_2). The myometrial strip was attached at each end to metal hooks and another hook was fixed to a transducer. Then the electrical signal has been recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant et al., 2002).

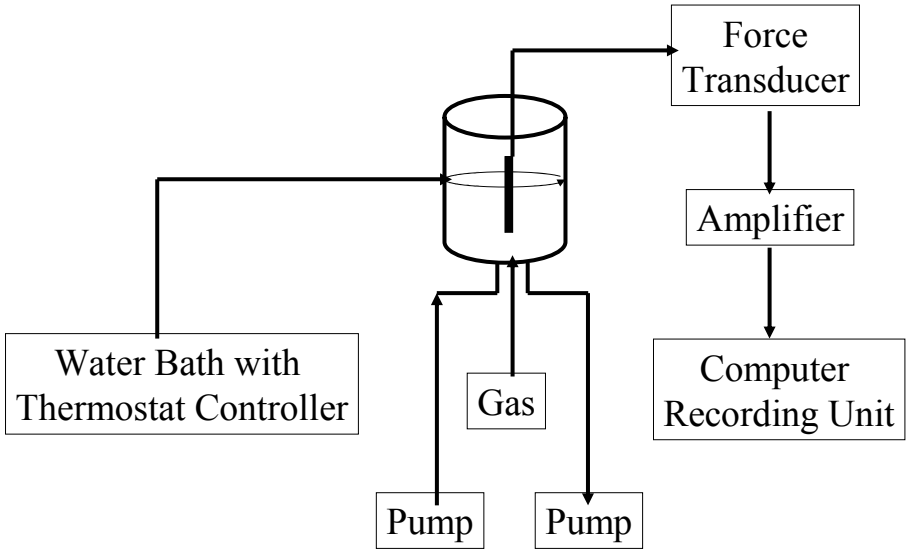

Figure 2.2 Schematic representation of the set up used for tension measurements.

2.4 Determination of PGE_2 Radio Immunoassay (RIA) and cAMP Enzyme Immunoassay (EIA)

2.4.1 Anti-Inflammatory RIA Assay

2.4.1.1 Cell Culture and Treatment

Immortalized mouse PGH-1 and PGH-2 null cells at the concentration (1×10^5 cells/ml) of Dubelcco's Modified Eagle Medium (DMEM) high glucose supplemented with hygromyein B (200 µg/ml), non essential amino acid (0.1 mM), L-glutamine (50 mg/l), ascorbic acid (0.05 mg/ml) and 10% fetal calf serum (FCS) were seeded into 96-well flat bottom tissue culture plates (83 µl/well). Cells were incubated 37°C in humidified incubator with 5% CO_2 for 7.2 hr. The cells were then washed with DMEM medium without FCS and pre-incubated for 30 min with 83 µl of serum-free DMEM medium containing vehicle or drugs. Following the pre-incubation

period, the medium was removed and the cells were immediately treated with serum-free medium containing vehicle or drugs and AA (20μM) or A 23187 (2 μM) for 30 min (Kirtikara et al., 1998). Culture supernatants were then collected from wells and analyzed for PGE_2 concentrations by radioimmunoassay (RIA).

2.4.1.2 PGE_2 Measurement

The RIA method used for measuring PGE_2 concentrations in the culture supernatant is based on the competition between PGE_2 in samples and ^3H labeled PGE_2 for anti-PGE_2 antibody binding sites. The assay was performed on ice as following. The supernatant was diluted with DMEM (1:10) for blank and zero % binding and added approximately 1.5 ml into 50 μl micro-centrifuge tube. Then, 50 μl of anti-PGE_2 antibody in RIA buffer (0.1 mM phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.1% sodium azide and 0.1% gelatin) was added to every tube except for the blanks, in which 50 μl RIA buffer was added. Subsequently, 50 μl of ^3H-PGE_2 (1.12 μci/ml), was added to each tube, vortexed briefly and incubated overnight at 4°C. Then, 100 μl at 2% charcoal-dextran suspension in RIA buffer was added to each tube. After 15 min incubation on ice, the tubes were centrifuged at 3,800 rpm (1,500g) at 4°C for 10 min. Supernatants were then transferred to new 1.5 ml micro-centrifuge tubes containing liquid scintillation cocktail, vortexed and counted for radioactivity. The resulting radioactive counts were used to calculate % binding of ^3H-PGE_2, which were then used for the estimation of PGE_2 concentrations from standard curves. This part of work was conducted by National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand.

2.4.2 cAMP EIA Assay

cAMP content of myometrial smooth muscle cells were measured by enzyme immunoassay (EIA). The uterine strips were incubated with 5mM $CaCl_2$ for 10 min then removed to Krebs' solution containing aminophylline (1 nM), ginger oil (50 µl/100 ml), 95% citral (2.2 mM), and 95% camphene (7.5 mM) at 37°C for 30 min. Post-incubated tissues with various treatments, the strips were rapidly frozen in liquid nitrogen and stored at -80°C until homogenized in 6% trichloroacetic acid (0.4 ml). The homogenate was centrifuged at 3000×g for 15 min. The supernatant was washed with 1.5 ml of water-saturated diethyl ether four times. The cAMP contents were assayed by using EIA kit (Amersham Pharmacia Biotech, Little Chalfont, UK). cAMP contents are presented as fmol/g wet weight.

2.5 Chemicals

All chemicals were purchased from Sigma® unless stated otherwise. Details of stock solutions were given in the individual chapter concerned. These stock solutions were prepared and kept as recommended by the producer. Dissolved vehicles (e.g. DMSO, ethanol) did not alter the myometrial contractile ability as judged by the peak tension, frequency of contractions, and contraction integral (see 3.4.1). The dilutions were made on the day of the experiment.

2.6 Data Analysis

Statistical Analysis of Tension Measurements

The result data were analyzed using Microcal Origin Software (Massachusetts, USA). Parameters that were measured include maximum tension development of each

contraction, the contraction integral (total tension developed in each contraction), contraction duration, and contraction frequency. IC_{50} values (concentrations) of multiple substance-inhibited 50% of myometrial contraction were calculated.

Data were then presented as mean ± s.e.m. and "*n*" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests or ANOVA and *P* values < 0.05 taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100%).

Statistical Analysis of PGE_2 Contents

The paired *t* test procedure was used to determine the differences in the PGE_2 levels between control samples of wild-type. COX-2 cells and among control samples and samples from cytokine-treated cells. Differences were considered significant if $p < 0.05$ (Kirtikara, Swangkul, and Ballon, 2001).

Statistical Analysis of cAMP Assay

Data were calculated as the average optical density (OD) for each set of replicate wells. The percent bound for each standard and sample (see below) was calculated.

$$\%B/B_0 = \frac{\text{(standard or sample OD-NSB OD)}}{\text{(zero standard OD-NSB OD)}} \times 100$$

Abbreviations:

OD = optical density

NSB = non specific binding wells

$\%B/B_0$ = the percent bound for each standard and sample

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2.1.5 Pharmacological Study of Ginger-Related Compounds

As shown in Table 2.1, the major active compounds found in ginger oil are the important role for evaluating potential mechanism to treatments.

Several studies have been investigated the effectiveness of ginger in the prevention and used in traditional medicine to treat such symptoms as inflammation, rheumatic disorders and gastrointestinal discomforts for long time (Peng, 1992; Aeschbach et al., 1994; Habsah et al., 2000; Surh, 1998; 2003). Its extract and major pungent principles, such as [6]-ginger and [6]-paradol, have recently been shown to exhibit a variety of biological activities including anticancer (Katiyar, Agarwak, and Makhtar, 1996; Surh and Lee 1998; Surh, 2003; Bode, Ma, Surh and Dong, 2001; Keum et al., 2002). The pungency inhibited on nitric oxide synthesis (Ippoushi, Itou, Azuma, and Higashio, 2003), COX-2 activity (Tjendraputra, Tran, Liu-Brennan, Roufogalis and Duke, 2001) and protecting neuronal cells from β-amyloid injure (D. Kim, and J. Kim, 2004). In contrast, Wei et at. (2005) reported that the study of Chinese ginger, gingerol-related compounds, has been shown to possess significant cytotoxicity against HL-60 cells (IC_{50} < 50 μM) and its cytotoxic activity was associated with the cell apoptosis.

The most important compounds, responsible for ginger's theraputic activity have been found such compounds include zingiberene, -ar-curcumene, β-sesquiphelandrene, α-pinene, camphene and citral (Mustafa, Srivastava, Jensen, 1993). Citral, a widely used natural ingredient, was added to foods and cosmetics as a flavoring and fragrance agent (Trasarti, Marchi and Apestegua, 2004). They have tested for citral conversion to menthols, Ni/Al-MCM-41; the best catalysis, which yielded menthol and menthol in the menthol mixture (Trasarti et al., 2004). In

addition, citral was used as a chemical intermediate in the synthesis of vitaminA, ionone and methylionone (Budavari, 1989). On the other hand, citral was selected for carcinogen studies because of its widespread use as a flavoring and fragrance ingredient. Citral administrered dermally has previously been studied skin severally irritating to albino angora rabbits, male Hartley guinea pigs, and humans (Basketter and Scholes, 1992; Cardullo, Ruszkowski, and Deleo, 1989; Motoyoshi, Toyoshima, Sato, and Yoshimura, 1979). Although it was positive in the local lymph node assay (Basketter and Scholes, 1992), citral also has been reported to induce benign and atypical prostatic hyperplasia in rats when applied dermally for one or more months (Engelstein, Shueli, Bruhis, Servadio, and Abramovici, 1996; Kessler, Keisari, and Servadio, 1998). In addition, citral at a high exposure (31,300 ppm); thymic atrophy in males and females has been revealed to inanition and the moribund condition of the rodent studies (Ress, 2003). There was no citral-related effect on the occurrence of either internally visible myometrial structural abnormalities, or uterine malformations.

Camphene is one of the attractive by its used on pharmaceutical and cosmetic applications. This is one of ginger compound obtained from pinene. Camphene hydrochloride has played an important historical role in the chemistry of the terpenes of carbonium ion rearrangements (Simonsen and Owen, 1957; Berson, 1963; Barlett, 1965; Eastman and Noller, 1953; Ingold, 1969). It was used in the manufacture of camphor and its related compounds (Comelli, Ponzi, E. N and Ponzi, M., 2005).

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CHAPTER III

EFFECTS OF GINGER OIL ON SPONTANEOUS AND PGs-INDUCED RAT MYOMETRIAL CONTRACTION

3.1 Abstract

Ginger rhizomes (*Zingiber officinale* Rocsoe) have been widely used not only as food, but also as medicine for intestinal disorders and relaxation of the smooth muscles. In addition, the essential oil and oleoresin from the ginger rhizomes were utilized as flavour additives in foods and beverages. The purpose of this study was to investigate the inhibitory effects of ginger oil on rat uterine contraction arising either spontaneously or by PGs ($PGF_{2\alpha}$ and PGE_2) stimulation and further investigated the underlying mechanisms.

Ginger rhizomes were provided and hydrodistilled to obtain the oil yield. Its structural constituents were then analyzed by GC/MS. Rats were humanely killed by asphyxiation with CO_2 and myometrial strips isolated. The effects of ginger oil on uterine contraction were measured and recorded. The results showed that ginger oil (10–150 μl/100 ml) inhibited spontaneous and PGs-induced contraction. Its effects were reversible upon elevation (from 2 mM to 5 mM) of external Ca^{2+} concentration. Its IC_{50} was 50 μl/100 ml. In summary, ginger oil is a potent inhibitor of myometrial contraction in rat uterus. This is probably due to the inhibition of L-type calcium channels.

3.2 Introduction

Zingiber officinale Roscoe, known as "Ginger," is widely used in foods as a spice around the world. Beside in Asia, ginger is used as household remedy for aromatherapy. In addition, it is used in medicine as a folk remedy to calm nausea associated with motion sickness (Lien et al., 2003) and to treat the digestive tract (Gruenwald et al., 2000). One of the most interesting properties of ginger rhizome is its inhibitory effect on rat ileal motility in *vitro* (Borrellia et al., 2004) and to relax the tracheal or ileal smooth muscle (Reiter and Brandt, 1985). An aqueous extract of a low dose of ginger rhizome (50 mg/kg) could be used in the rats as an cholesterol-lowering, antithrombotic, anti-inflammatory agent inhibitory effect on platelet aggregation. Furthermore, it has been reported that the levels of serum PGE_2 were significantly reduced in the presence of ginger (Srivastava, 1984, 1986; Thomson et al., 2002).

In vitro experiment, shogoals and 6-, 8-, and 10-gingerols, isolated from the methanolic extract of *Z. officinale* rhizome exhibited anti-emetic principles (Kawai et al., 1994). Both 6-shogaol and 6-gingerol have been shown to have a number of pharmacological activities including antipyretic, analgesic, antitussive and hypotensive effects (Suekawa et al., 1986). Moreover, anti-inflammatory data were obtained from the metabolic profiling analysis of *Z. officinale* samples. The oil derived from different origins show no qualitative differences in major volatile compounds, [6]-, [8]-, and [10]-gingerols (Park et al., 1998). The most active anti-inflammatory components were evaluated (Park et al., 1998; Young et al., 2005). The ability of all extracts from the *Zingiber* species was shown to inhibit LPS-induced PGE_2 and TNF-α production (Jiang et al., 2006). In addition, ginger has shown to

exhibit antithrombotic activity and inhibit prostaglandin-E_2 from arachidonic acid, gingerol, and dehydroparadol favored the inhibition of cyclooxygenase (Srivastava, 1984, 1986; Flynn et al., 1986).

However, the study of the effects of ginger constituents on the uterine contraction has not yet been elucidated. Therefore, the aims of this chapter were to 1) demonstrate the effects of ginger oil on spontaneous contractions and 2) $PGF_{2\alpha}$-and PGE_2-induced contractions in isolated rat myometrium in the presence or in the absence of external Ca^{2+} concentration. In addition, its effect on Ca^{2+}- CaM-MLCK pathway and non Ca^{2+}- CaM-MLCK pathway was investigated.

3.3 Materials and Methods

3.3.1 Chemicals and Physiological Solution

All chemicals were purchased from Sigma® unless state otherwise. Agonists/antagonists for the investigation of physiological pathways used were; $PGF_{2\alpha}$, PGE_2, nifedipine, and BSA. $PGF_{2\alpha}$ and PGE_2 were dissolved in ethanol at a concentration of 1 μM. Bovine serum albumin (BSA, 5%) was dissolved in distilled water. Nifedipine was dissolved in DMSO at a concentration of 10 μM.

3.3.2 Ginger Oil Preparations

As described in 2.1.3, a stock solution of the oil was dissolved by hexane in Krebs' solution just before use. The final concentration of hexane in any dilution was less than 0.15%.

3.3.3 Myometrial Tissue Preparations

Tissue preparations are essentially the same as those described in Chapter II. Non-pregnant Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand. Myometial tissue preparations were dissected and provided for tension measurements as those described in 2.2.2.

3.3.4 Measurements of Tension

The uterine strip was mounted vertically under resting tension of 1g in a single chamber (25 ml) tissue bath connected to a force transducer (as described in 2.3).

The organ bath contained Krebs' solution maintained at pH 7.4, temperature of 37 ^{o}C, and gassed with carbogen (95% O_2 and 5% CO_2). The myometrial strip was attached at each end to metal hooks and another hook was fixed to a transducer. The electrical signal has been recorded from the transducer and converted to the digital signal on a computer using Chart software (Kupittayanant, 2003).

3.3.5 Data Analysis

Data were presented as mean ± S.E.M. and "*n*" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests or ANOVA and p values < 0.05 taken to be significant. Results were then

expressed as percentages of control contractions (i.e. the control is 100%, as described in 2.6).

3.4 Results

3.4.1 Effects of Vehicle on Spontaneous Contraction

To test whether the solvent, hexane, used as vehicle in the following experiments did not affect the uterine contractility, hexane at various concentrations was applied to spontaneous contraction. As shown in Figure 3.1, hexane at the concentration of 50 µl/100 ml did not affect the contractility in term of the amplitude, frequency, and duration of spontaneous contraction.

The spontaneous contraction of uterine strip adding vehicle hexane level (50 µl/100 ml), the solvent used in the experiment did not affected the contractile activity and was not significantly different ($p > 0.05$) amplitude, frequency, and AUC (97.06±3.51, 97.00±4.0 and 99.86±0.51, respectively).

Figure 3.1 The effects of hexane on spontaneous contraction.

3.4.2 Effects of Ginger Oil on Spontaneous Contraction

To determine the half inhibitory effects (IC_{50} values) of ginger oil, ginger oil at various concentrations (10-150 μl/100 ml) was applied to spontaneous contraction. Its effects are shown in Fig. 3.2 and Table 3.1.

The IC_{50} of ginger oil was found at 50 μl/100 ml (Fig. 3.3A and Table 3.1). The mean value of contraction amplitude was 49.03±3.10% and the area under the curve (AUC) was 50.69±3.66%, compared with 100% of the control. This concentration was used throughout the study. However, this concentration ginger oil had little effect on the frequency of the contraction. The frequency of the contraction was decreased to 77.96±1.53%, compared to the control (100%).

3.4.3 Effects of Ginger Oil on Spontaneous Contraction in the Continued Presence of High Ca^{2+}

An increase in $[Ca^{2+}]_i$ is generally considered to control the contractile activity in myometrium (Word, 1995). Various Voltage-gate Ca^{2+} channels, as well as the release of Ca^{2+} from intracellular Ca^{2+} store are involved (Berridge, 1997). During periods of myometrial contraction, each of these processes may contribute to the overall rise in $[Ca^{2+}]_i$. Therefore, an increase in L-type Ca^{2+} channel activity (Hurwitz, 1986) in the continued presence of ginger oil is one of the ways to examine the effect of ginger oil on Ca^{2+} signaling.

When 50 μl/100 ml ginger oil was applied to spontaneous contraction, it gradually decreased the amplitude of the contractions (Fig. 3.3A). To test whether this inhibitory effect of ginger oil was due to an inhibition of L-type Ca^{2+} channels, the concentration of external Ca^{2+} was elevated while ginger oil was continued present.

As seen in Figure 3.3B, addition of 5 mM $CaCl_2$ reversed the effect of ginger oil. The amplitude of the contraction was increased to 113.46±5.07%, compared with the preceeding contractions with ginger oil alone (Fig. 3.3).

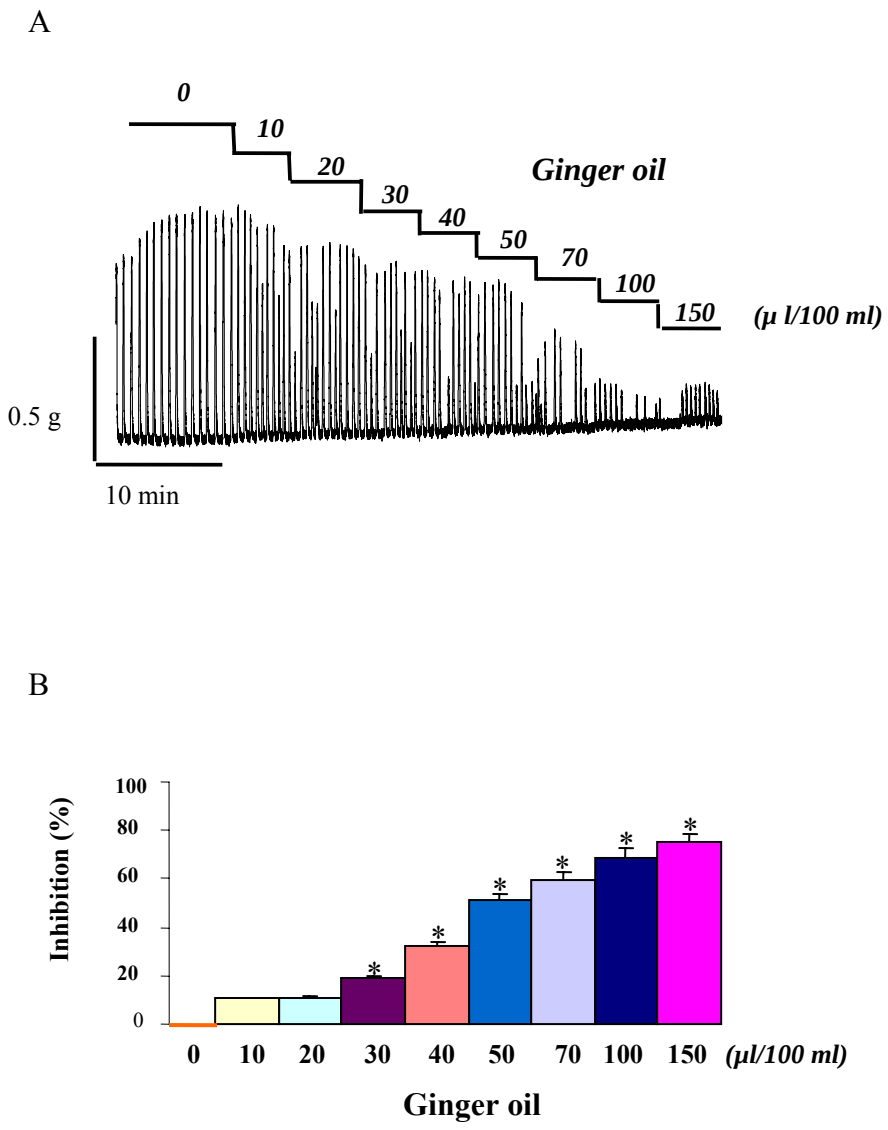

Figure 3.2 The effects of ginger oil at various concentrations on spontaneous contraction.

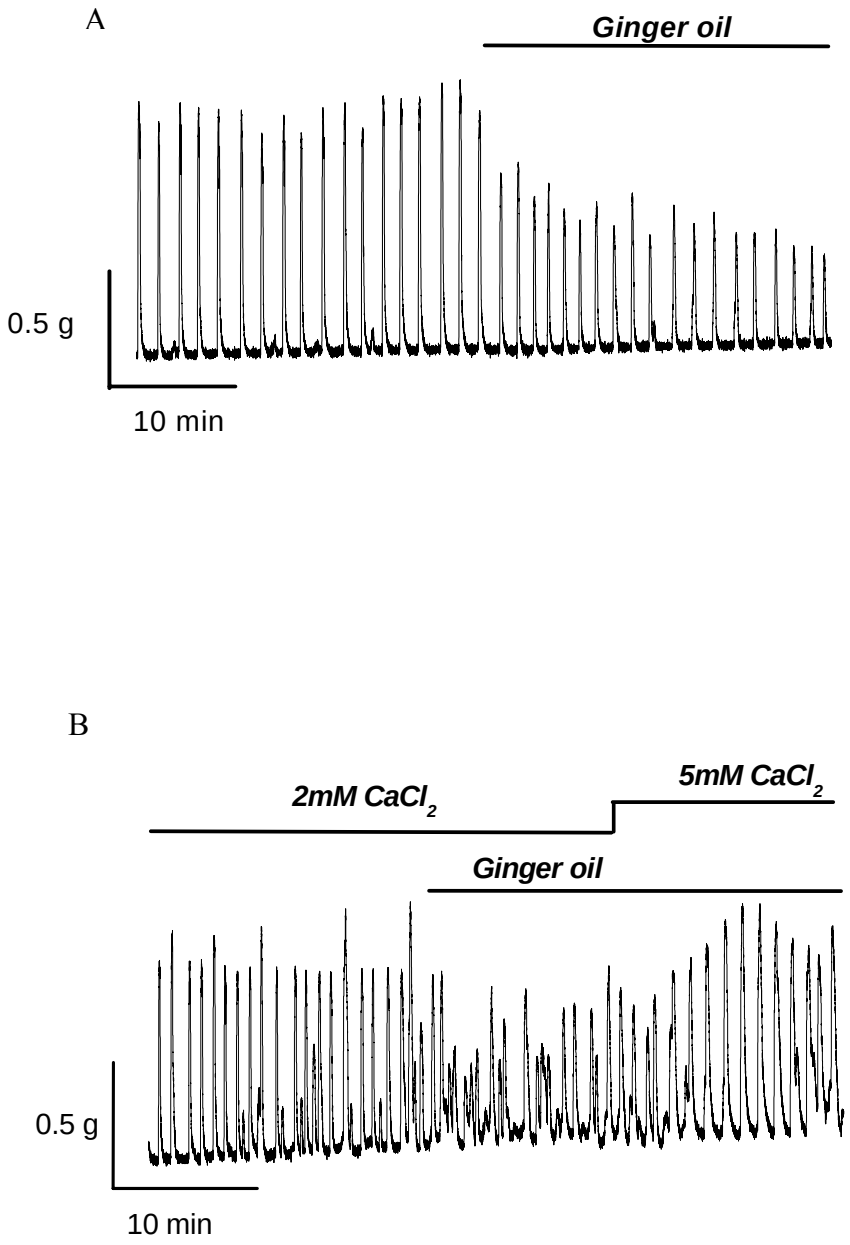

Figure 3.3 The effect of ginger oil (50 µl/100 ml) on spontaneous contraction. (A) and the effect of ginger oil (50 µl/100 ml) in the presence of 5 mM (B) $CaCl_2$ is shown (B).

3.4.4 Effects of Ginger oil on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction

It has been shown that $PGF_{2\alpha}$ and PGE_2 can increase uterine contraction by inducing Ca^{2+} entry via L-type Ca^{2+} channels and releasing Ca^{2+} from the SR store (Luckas et al., 1999). As can be seen in Figure 3.4, application of either 1 μM $PGF_{2\alpha}$ or 1 μM PGE_2 causes a significant increase in the contractions of spontaneously contracting uterus. With $PGF_{2\alpha}$, the amplitude, the frequency, and AUC of the contraction was increased to 128.42±5.13%, 128.37±3.19%, and 156.21±2.96%, compared to 100% of the control. With PGE_2, the amplitude, the frequency, and AUC of the contraction was increased to 128.17±5.48%, 154.89±2.38%, and 179.61±2.48%, compared to 100% of the control (Table 3.2). Having demonstrated the effects of PGs on spontaneous contraction, the effects of ginger oil on $PGF_{2\alpha}$-and PGE_2-induced contraction were investigated. The effects are summarized in Table 3.2.

The effects of ginger oil on PGs-induced contraction were investigated (Fig. 3.5). When 50 μl/100 ml ginger oil was applied in the continued presence of either $PGF_{2\alpha}$ or PGE_2, it significantly inhibited the contraction. With $PGF_{2\alpha}$, the amplitude, frequency, and AUC of the contraction were reduced to 56.39±5.34%, 81.24±3.68%, and 56.49±4.28%, compared to 100% of the control (Table 3.2). With PGE_2, the amplitude, frequency, and AUC of the contraction were reduced to 50.65±6.99%, 58.64±0.10%, and 57.61±2.48%, compared to 100% of the control.

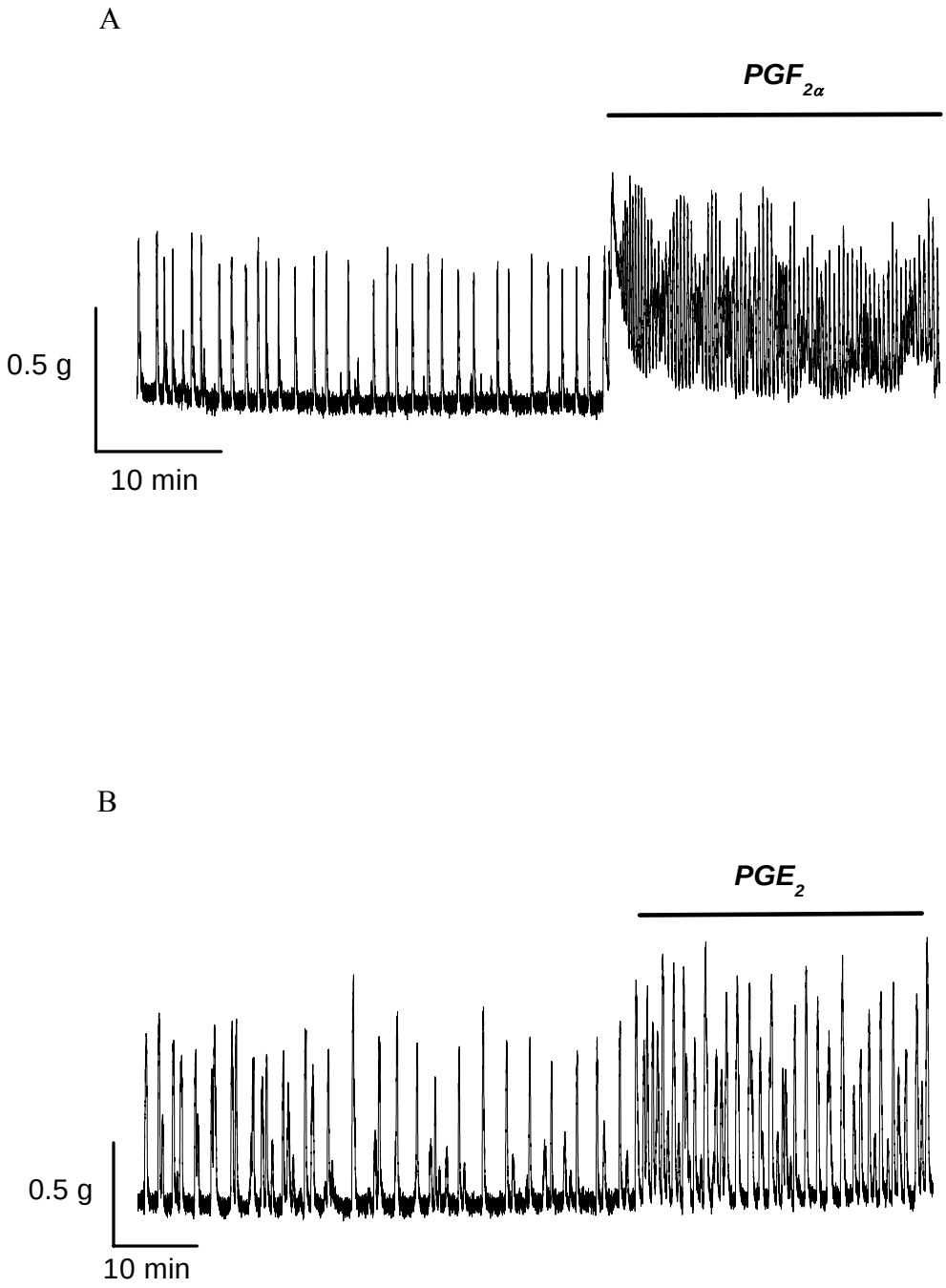

Figure 3.4 The effects of $PGF_{2\alpha}$ (A)-and PGE_2 (B)-induced contractions.

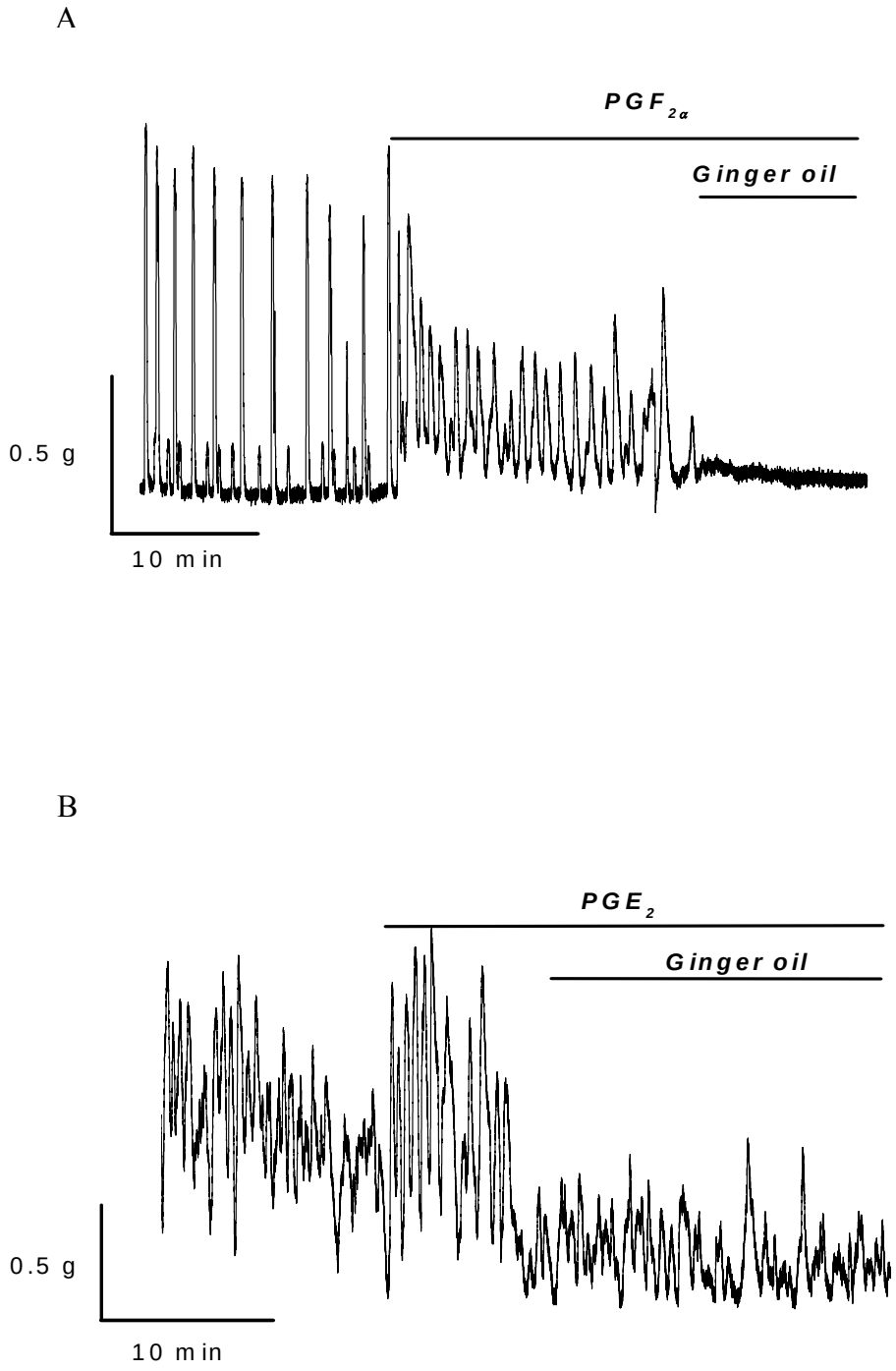

Figure 3.5 The effects of ginger oil on PGs-induced contractions. The applications of ginger oil on $PGF_{2\alpha}$ (A)-and PGE_2 (B)-induced contraction are shown.

3.4.5 Effects of Ginger Oil on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction in the Continued Presence of High Ca^{2+}

As shown in Figure 3.5, ginger oil inhibited both $PGF_{2\alpha}$ and PGE_2 contraction. Next, the underlying mechanisms were investigated. To test whether the inhibitory effect of ginger oil on the agonist-induced contraction was via the inhibition of L-type Ca^{2+} channels, the effects of ginger oil were tested in the continued presence of high Ca^{2+}.

As seen in Figure 3.6, the effects of ginger oil can be reversed upon an increase in external Ca^{2+} concentration. This was also the case for PGE_2 (data not shown). The effect of ginger oil on $PGF_{2\alpha}$-and PGE_2-induced contraction in the presence of high Ca^{2+} are summarized in Table 3.3.

3.4.6 Effects of Ginger Oil on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction in the Absence of External Ca^{2+}

It has been reported that PGs can cause small contraction independent of external Ca^{2+}, presumably via non-Ca^{2+}-CaM MLCK pathway (Luckas et al., 1999). As the previous report, in the present study showed that both $PGF_{2\alpha}$ and PGE_2 caused the small rise in contraction when external Ca^{2+} entry was inhibited by nifedipine (Fig. 3.7A and 3.8A, respectively). To test whether, ginger oil could inhibit this contraction, it was applied in the continued of these agonists.

As can be seen in Figure 3.7B and C, the application of ginger oil before and after the application of $PGF_{2\alpha}$ abolished the contraction. This was also the case of PGE_2 (Fig. 3.8B and C).

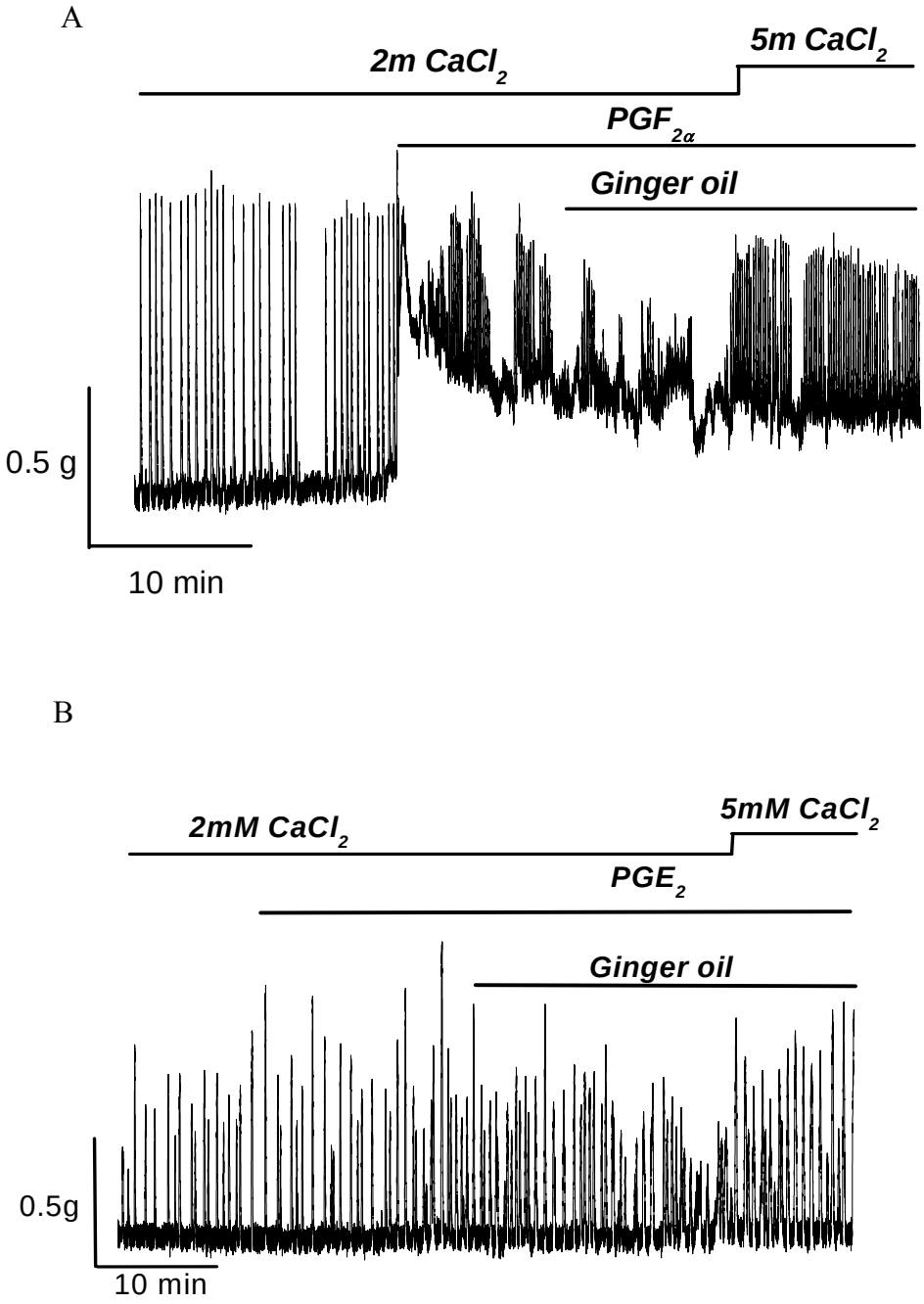

Figure 3.6 The effects of ginger oil on PGs-induced contractions in the presence of high Ca^{2+} concentration (5 mM $CaCl_2$). (A) An application of ginger oil on $PGF_{2\alpha}$-induced contraction is shown. (B) An application of ginger oil on PGE_2-induced contraction is shown.

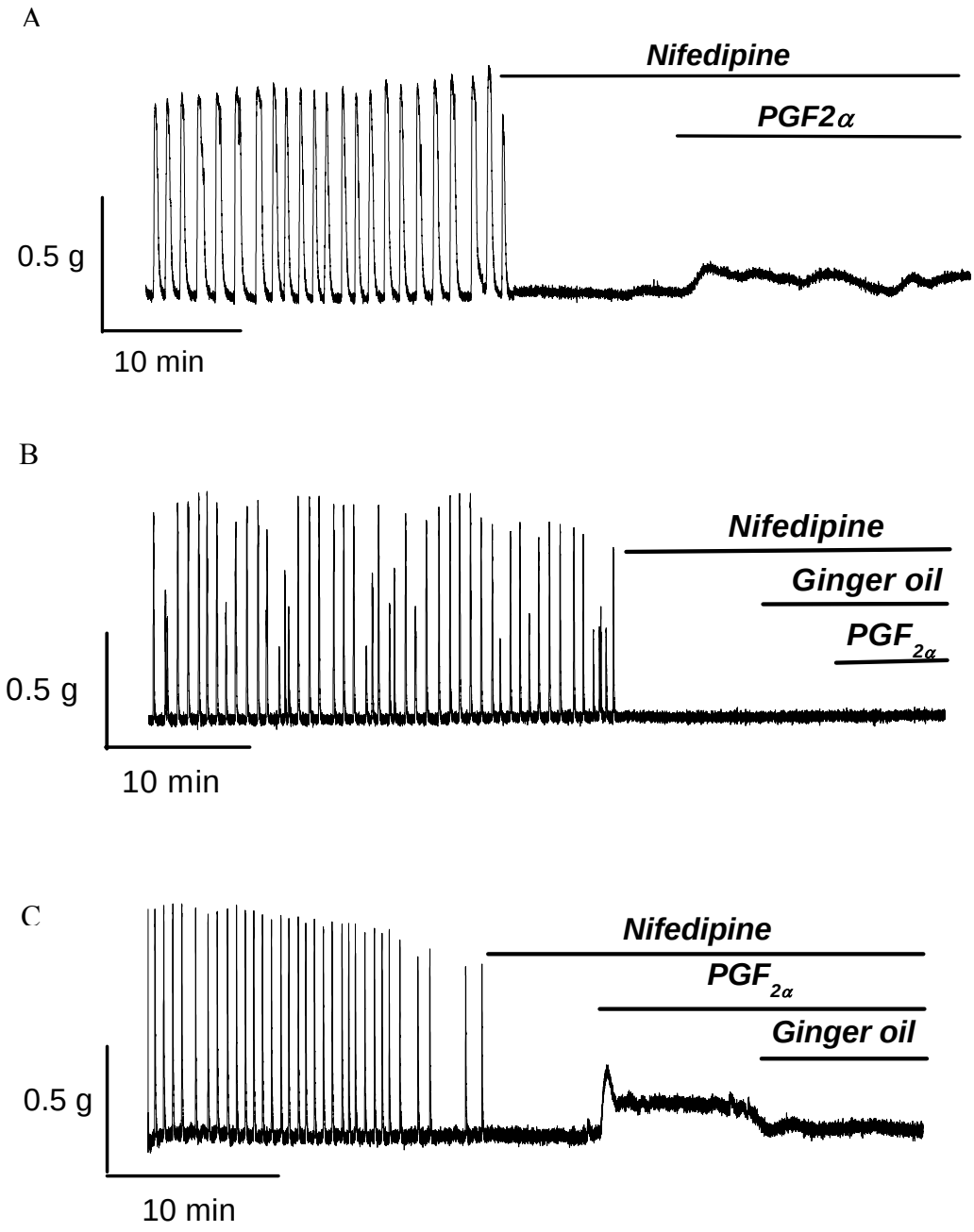

Figure 3.7 The effects of ginger oil on $PGF_{2\alpha}$-induced contraction in the presence of nifedipine. (A) $PGF_{2\alpha}$ (1μM)-induced contractions is shown. The application of ginger oil added before (B) and after (C) $PGF_{2\alpha}$-induced contractions is shown.

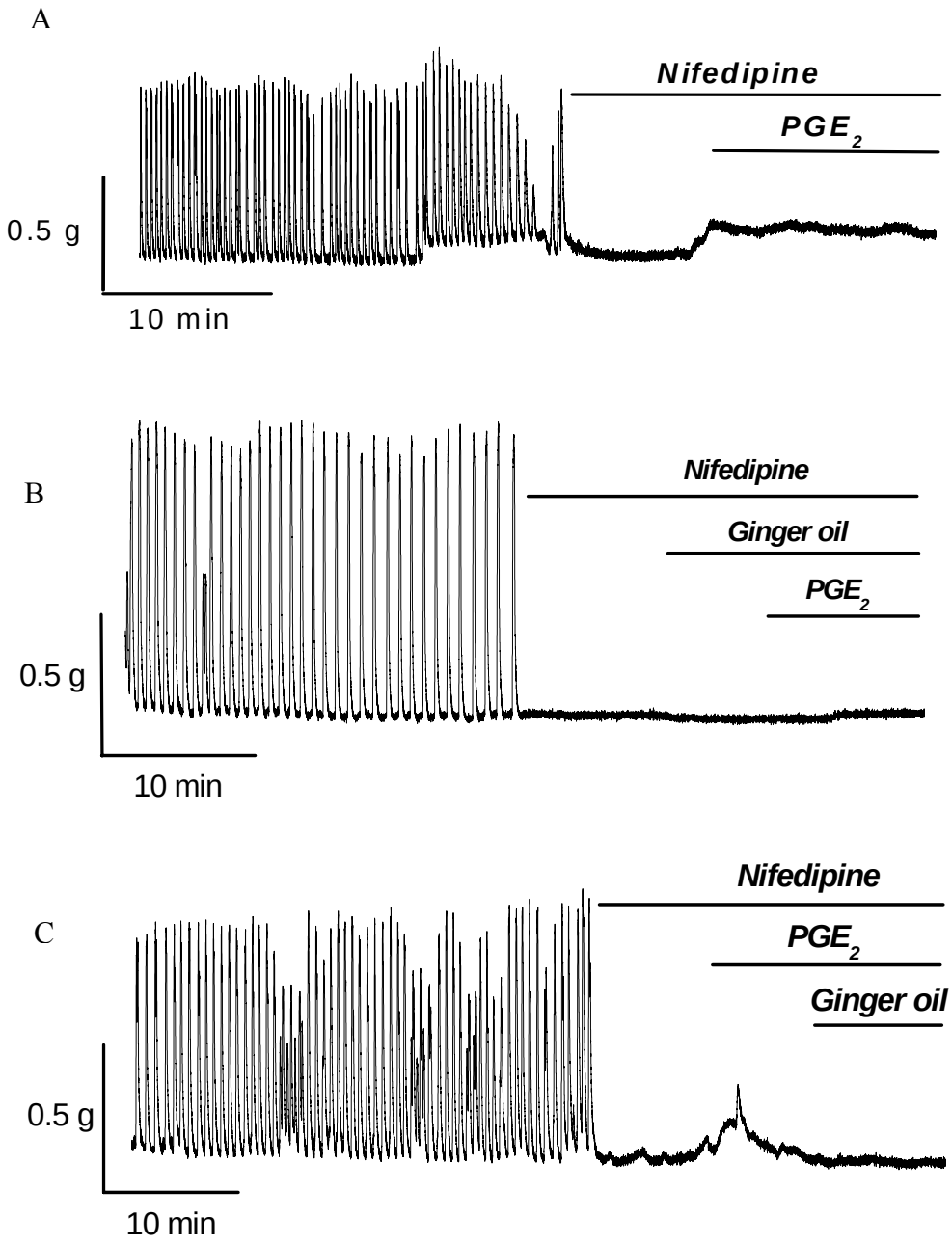

Figure 3.8 The effects of ginger oil on PGE_2-induced contraction in the presence of nifedipine. (A) PGE_2 (1μM)–induced contractions is shown. The application of ginger oil added before (B) and after (C) PGE_2-induced contractions is shown.

3.4.7 Effects of BSA on Ginger Oil

As shown in Chapter II, free fatty acids were found as a major constituent of ginger oil and that it has been reported to alter the uterine contraction (Babiychuck et al., 2002) leading to relaxation. Next, the effect of BSA on ginger oil was investigated.

As shown in Figure 3.9 an application of BSA reversed the inhibitory effect of ginger oil.

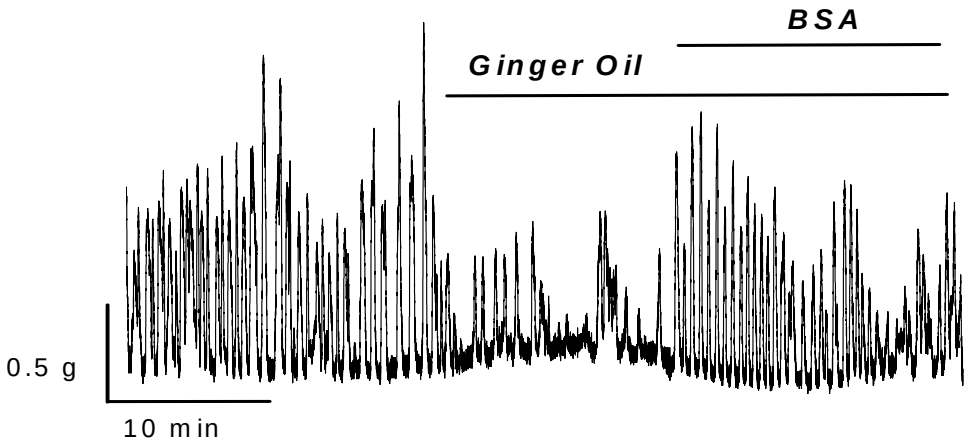

Figure 3.9 The effect of ginger oil on spontaneous contraction in the continued presence of BSA.

3.5 Discussion

The data indicated that the effects of ginger oil on rat myometrial contractile activity may occur by inhibitory of Ca^{2+} influx via the L-type Ca^{2+} channels. This was also the case for both spontaneous and PGs-induced contractions.

Ginger oil statistically significant ($p < 0.05$) effect in reducing contractile activity of rat myometrium. However, low dose of ginger oil (10 – 20 μl/100 ml) did not more effectively and not significantly ($p > 0.05$) different amplitude, frequency and AUC than the spontaneous contraction (control) in non-pregnant rat uterus. Although, the inhibitory effect of ginger oil (as seen in Fig. 3.9) could be reduced to half-maximum contraction, it was induced by BSA similarly to the control.

An increase the external Ca^{2+} concentration can reversed the effect of ginger oil on spontaneous contraction to compare with the control. Furthermore, the effect of ginger oil in the presence of high Ca^{2+} concentration and in PGs-induced contractions are also returned to a rise the recovering contractility (Fig. 3.6). Because of these agonists, PGs are also most likely to play an important role in these conduction to induce Ca^{2+} from the SR store via IP_3 binding to receptors on the SR membrane, known as IP_3–induced Ca^{2+} release (IICR; Berridge, 1997). Thus, it has been clearly demonstrated that agonists induced the release of Ca^{2+} and contraction had been rapidly abolished by nifedipine (10 μM) due to inhibit the L-type Ca^{2+} channels (Coleman et al., 2000; Kupittayanant, 2000). As a result of the same concentration of PGs-induced contraction in the absence (nifedipine) of external Ca^{2+} did not reverse the inhibitory effect of ginger oil (50 μl/100 ml, Fig. 3.7B, C). Although, the lone of PGs in this condition also increased amplitude and AUC of spontaneous contraction

but did not induce spontaneously return to contraction while adding ginger oil (50 μl/100 ml) in the absence of Ca^{2+} concentration (Figs. 3.7B, 3.8B).

These parameters are similarly shown to the effect of *Melisa officinale* extract, the strong constituent of citral from essential oil could reduce contraction on the rat isolated ileum in the presence of high KCl (Sadraei et al., 2003). Such the result of this experiment, citral has been the major compound that found in hydro-distillation from fresh ginger rhizome.

In conclusion, the inhibitory effect of ginger oil may be elucidated to reduce both spontaneous and $PGF_{2\alpha}$/PGE_2-induced contractile activity via closing the L-type Ca^{2+} channels with citral. In addition, it is clear that ginger oil has effect on Ca^{2+} rise in smooth muscle and also cause to specific effect on Ca^{2+} CaM-MLCK pathways.

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CHAPTER IV

EFFECTS OF CITRAL AND CAMPHENE ON SPONTANEOUS AND PGs-INDUCED RAT MYOMETRIAL CONTRACTION

4.1 Abstract

As citral and camphene are the most constituents found in ginger oil and are commercial available, the aims of this Chapter were therefore to study the effects of their agents on uterine contractility. The effects on both spontaneous and PGs-induced contraction were particularly investigated. The results showed that the effects of citral and camphene were similar indistinguishable to those of ginger oil. Thus, they decreased uterine contractions, irrespectively of how they were produced, via the inhibitory of L-type Ca^{2+} channels.

4.2 Introduction

The effects of pure citral and camphene have not been investigated. As they were abundant found in ginger oil (Table 4.1). It is worth investigating whether the effect of ginger oil found in Chapter III was due to the effect of camphene or citral.

4.2.1 Citral

Citral is a naturally-occurring mixture of the *cis* (neral) and *trans* (geranial) genometric isomers of 3,7-dimethyl-2,6-octadien-l-al. It imparts a strong "lemony" scent of edible vegetables and fruits, including lemon, lime, orange, grapefruit, tomatoes, and ginger (Miyazawa and Kameoka, 1988). In addition, citral by far is the highest constituent (75% - 85%) of lemongrass (*Cymbopogon citratus*) oil, as well as of ginger oil (Wohlmuth et al., 2006).

Several previous investigations have been reported that citral was found to possess anticancer effect against prostate gland tumor in various strains of rats (Scolnid et al., 1994). It was known to possess antiseptic, antimicrobial, anti-inflammatory, carminative, diuretic and central nervous system stimulating effects (Carbajal et al., 1989). Besides, the antioxidant activity of citral was tested in human intestinal homogenates *in vitro*. It inhibited the conversion of β-carotene to retinoic acid by preventing the oxidation process (Wang et al., 1992). It was found that citral is devoid of major toxicity and carcinogenic potential in both mice and rats (Ress et al., 2003) and that it is devoid of mutagenic effect *in vitro* models (Viniketkumnuen et al., 1994).

4.2.2 Camphene

Camphene is one of volatile oil consisted of mainly monoterpene in ginger oil. It has several pharmacological properties. For example, Iranian folk medicine has reported its antispasmodic, carminative, stomachic effects. The oil has been used as a spasmolytic remedy for abdominal cramps in some parts of Iran (Sdraei et al., 2003). According to its recommended clinical application it was tested on the respiratory

system as well as by its application on epilated skin. In addition, its combination with menthol exerts spasmolytic activity in intestinal smooth muscle by blocking calcium channels (Hawthorn et al., 1988), and attenuates bronchoconstriction and cough by combined anaesthetic action on sensory nerves and bronchial smooth muscle (Wright et al., 1997). Essential oil contained camphene was given twice daily for four weeks has been shown to improve asthma control (Tamaoki et al., 1995).

There are number of pungent compounds found in fresh ginger rhizomes as shown in Figure 4.1 and Table 4.1. These include citral (14.19% geranial, 10.00% neral) and camphene (7.74%), which are commercially available compounds. The main aims of this Chapter were to study the effects of citral and camphene on uterine smooth muscle contraction. The effects on spontaneous or PGs-induced contractions were particularly examined as well as the effects of these compounds in the presence or the absence of external Ca^{2+} concentrations.

4.3 Materials and Methods

4.3.1 Identification of the Ginger Oil Constituents

The extracted oil was analyzed for the constituents by gas chromatography-mass spectrometry (GC-MS), the analysis was performed on a Hewlett-Packard 5973 (IE) MS selective detector coupled with Hewlett Packcard 6890 gas chromatography equipped with a cross-linked 5% PHME siloxane HP-5MS capillary column (30 m×2.25 mm; film thickness, 0.25 μm). The gas chromatography condition was as follows: carrier gas, helium with a flow rate of 1.0 ml/min; column temperature, 50°C at 6°C/min; injector temperature, 250°C; volume injected, 1.0 μl of the oil; split ratio, 250:1.

Identification of constituents was based on computer matching against the library spectra with components of known constituents. MS literature data and evaluation of fragmentation patterns of compounds were confirmed by their gas chromatography retention time indices of authentic reference compounds where possible. This part of work was conducted by Science and Technology Service Center, Chaing Mai University, Thailand.

4.3.2 Chemicals and Physiological Solution

All chemicals were purchased from Sigma® unless state otherwise. Agonists/antagonists for the investigation of physiological pathways were used, including citral (95%), camphene (95%), $PGF_{2\alpha}$, PGE_2, nifedipine, and BSA. Citral and camphene were dissolved by hexane in Krebs' solution just before used. The final concentration of hexane in any dilution was less than 0.15%. $PGF_{2\alpha}$ and PGE_2 were dissolved in ethanol at a concentration of 1 μM. BSA (5%) was disolved in distilled water. Nifedipine was dissolved in DMSO to give a final concentration of 10 μM.

4.3.3 Myometrial Tissue Preparations

Tissue preparations are essentially the same as those described in Chapter II. Non-pregnant Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT), Thailand.

Myometial tissue preparations were dissected and provided for tension measurements as those described in 2.2.2.

4.3.4 Measurements of Tension

The uterine strips were mounted vertically under resting tension of 1g in a single chamber (25 ml) tissue bath connected to a force transducer (as described in 2.3). The strip was allowed to contract spontaneously. Any chemical used in the study was applied after an equilibrium period was taken. The data were recorded as the electrical signal from the transducer and converted to a digital signal on a computer using Chart software.

4.3.5 Data Analysis

Data were then presented as mean ± S.E.M. and "*n*" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests or ANOVA and *P* values < 0.05 taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100%).

4.4 Results

4.4.1 Chemical Constituents of Ginger Oil

The ginger oil was found to contain high mono-and sesqui-terpenes and others as described in the Table 4.1.

Table 4.1 Chemical constituents of ginger oil identified by GC-MS analysis.

No.	Compound identified	Relative constituents (%)	Retention time (min)
1.	α-Zingiberene	16.21	17.88
2.	Geranial (E-citral)	14.49	13.03
3.	Neral (Z-citral)	10.00	12.35
4.	Camphene	7.74	5.70
5.	β-Phellandrene	7.44	7.43
6.	α-Farmesene	6.80	18.09
7.	β-Sesquiphellandrene	5.70	18.45
8.	Curcumene	4.71	17.61
9.	Eucaryptol	3.33	7.47
10.	α-Pinene	2.92	5.40
11.	Geraniol	2.85	12.62
12.	α-Bisabolene	2.42	18.13
13.	β-Cubebene	2.02	17.98
14.	β-Citonellol	1.76	12.02
15.	β-Myrcene	1.60	6.54
16.	Camphol	1.00	10.63
17.	Germacrene B	0.99	19.18
18.	Linalool	0.97	9.02

Table 4.1 (*continued*).

No.	Compound identified	Relative constituents (%)	Retention time (min)
19.	α-Terpineol	0.93	11.20
20.	Geranyl acetate	0.84	15.44
21.	Sesquisabinene hydrate	0.71	2 0.54
22.	Zingiberenol	0.68	20.21
23.	Nonyl	0.59	13.48
24.	Unknown	0.56	21.61
25.	Unknown	0.47	10.94
26.	β-citronellal	0.46	10.25
27.	s-Heptyl alcohol	0.44	4.71
28.	l-Phellandrene	0.42	6.86
29.	β-Pinene	0.37	6.28
30.	Citronellol acetate	0.37	14.78
31.	α-Terpinolene	0.21	8.77

4.4.2 Effects of Citral and Camphene on Spontaneous Contraction

Effects of Citral on Spontaneous Contraction

Under control conditions, spontaneous contractions of consistent amplitude AUC and frequency could be recorded for several hours; allowing different concentrations of citral to be tested in the rat uterus. Citral, in a dose-dependent response (0.5 to 8.3 mM), significantly inhibited the myometrail contraction arising spontaneously (Fig. 4.2A and Table 4.2, n = 5). The IC_{50} of citral occurred at the concentration of 2.2 mM. Thus this concentration was used throughout the experiments.

Effects of Camphene on Spontaneous Contraction

The effects of camphene on spontaneous contraction was tested at various concentrations under the same condition (Fig. 4.2B and Table 4.3). As could be observed, the effects of camphene was not as strong as of citral. The IC_{50} occurred at the concentration of 7.5 mM (Fig. 4.2B and Table 4.3, n = 5). A typical effects of camphene on spontaneous contraction is shown in Figure 4.2B. Camphene (7.5 mM) decreased the contraction amplitude, frequency, and AUC to 53.42±3.52%, 50.64±2.52%, and 53.42%±3.21%, respectively (Fig. 4.3B).

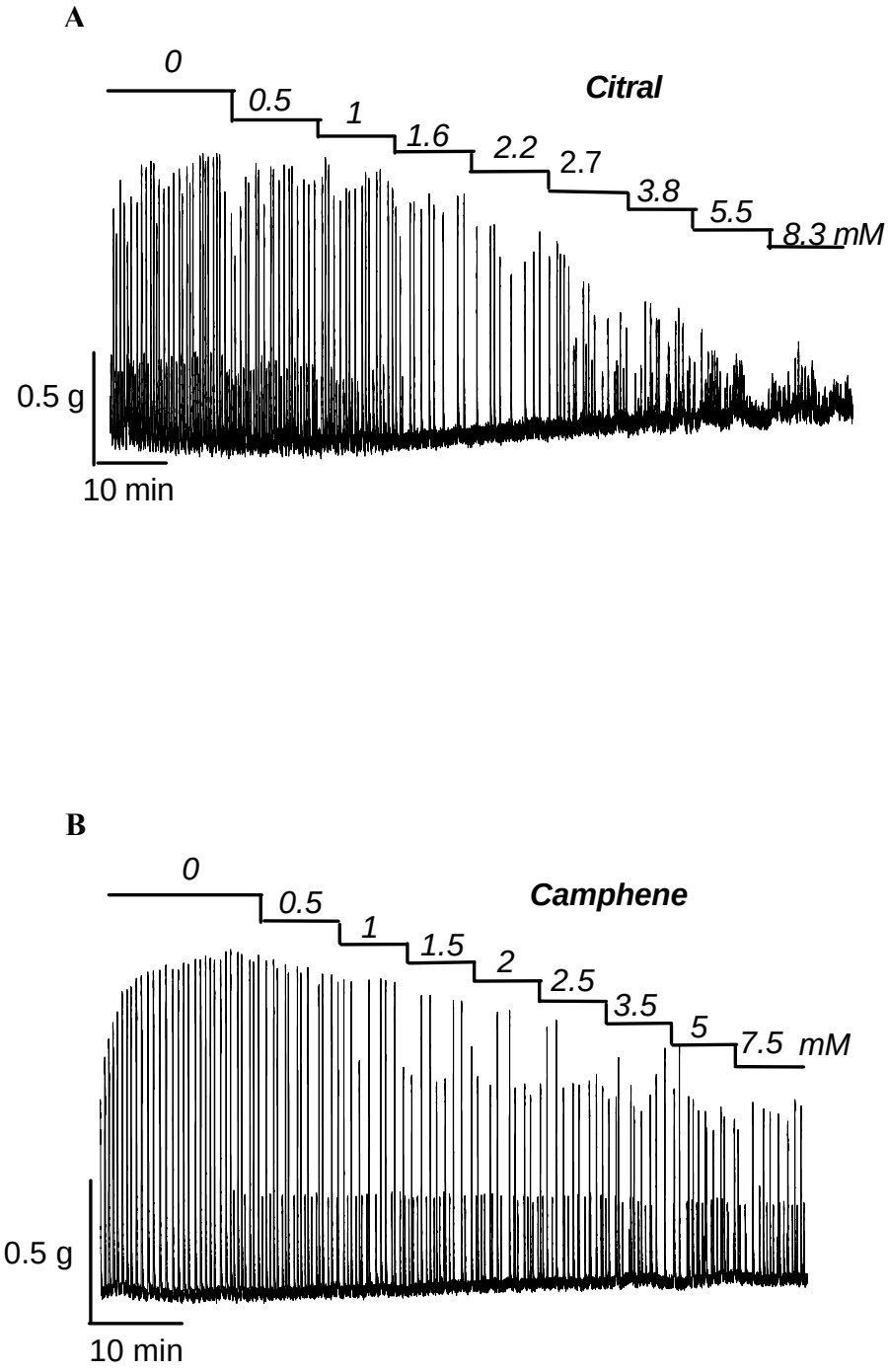

Figure 4.2 The effects of citral and camphene on spontaneous contraction.

The application of (0.5 – 8.3 mM) citral (A) and (0.5 – 7.5 mM) camphene (B) to spontaneous contractions is shown.

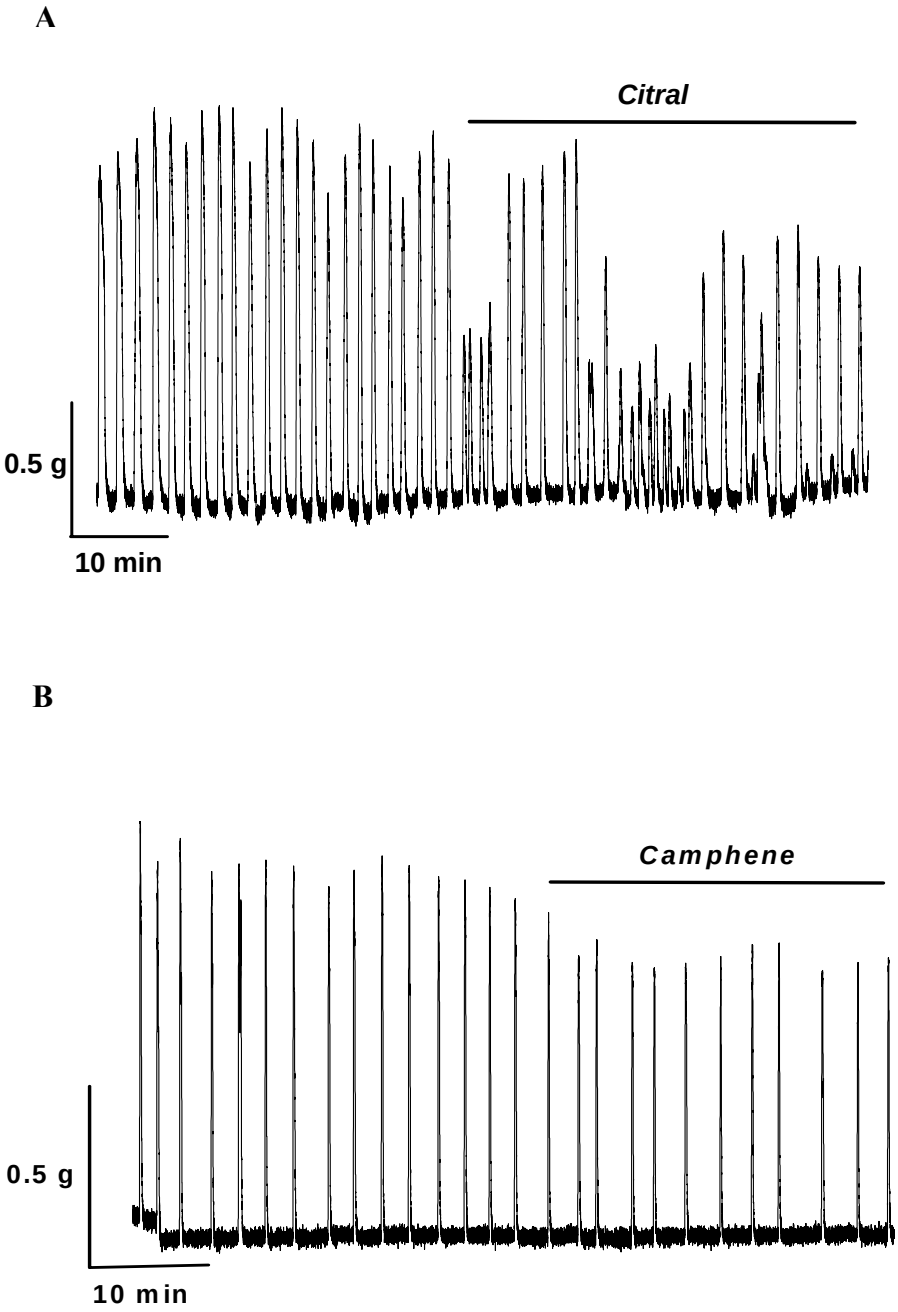

Figure 4.3 The effects of citral and camphene on spontaneous contraction.

The application of (2.2 mM) citral (A) and (7.5 mM) camphene (B) to spontaneous contractions is shown.

4.4.3 Effects of Citral and Camphene on Spontaneous Contraction in the Continued Presence of High Ca^{2+}

Effects of Citral on Spontaneous Contraction in the Continued Presence of High Ca^{2+}

The experiments were conducted to determine whether a rise in $[Ca^{2+}]_i$ could reverse the inhibitory effects of citral on spontaneous contractions. As can be seen in Figure 4.4A, an increase in external $[Ca^{2+}]$ from 2 to 5 mM into Krebs' solution increased the mean values of contraction amplitude to 108.93±3.74%, contraction frequency to 141.29±1.06%, and AUC to 135.85±1.88% ($p < 0.05$, n = 5); compared with 100% of the control. The effects of high Ca^{2+} were summarized in Table 4.4.

Effects of Camphene on Spontaneous Contraction in the Presence of High Ca^{2+}

As shown in Figure 4.4B, camphene at IC_{50} (7.5 mM) was applied for 30 min and again spontaneous contractions decreased. In Figure 4.4B and Table 4.4, after adding 5 mM $CaCl_2$, the inhibitory effects of camphene were also reversed. The mean values of contraction amplitude were increased to 113.72±3.65%, contraction frequency to 128.62±3.42% and AUC to 135.76±2.68% ($p < 0.05$, n = 5); compared with the control (100%).

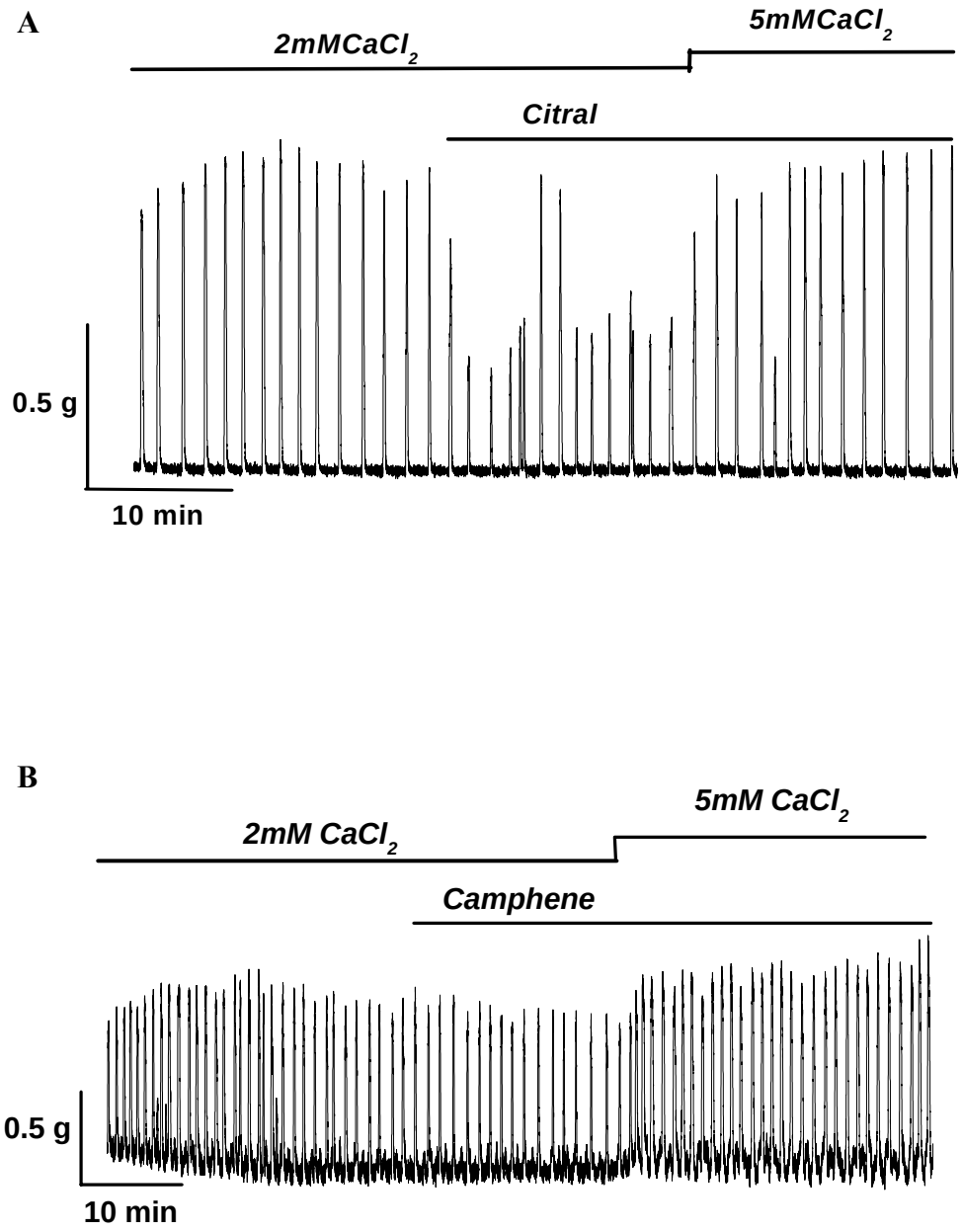

Figure 4.4 The effects of citral and camphene on spontaneous contraction.

(A) The application of (2.2 mM) citral in the presence of 5 mM $CaCl_2$ is shown. (B) The application of camphene in the presence of 5 mM $CaCl_2$ is shown.

4.4.4 Effects of Citral and Camphene on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction

Effects of Citral on $PGF_{2\alpha}$- and PGE_2-Induced Uterine Contraction

The present studies were designed to investigate the effects of agonists ($PGF_{2\alpha}$ and PGE_2)-induced myometrial contractions (Fig. 4.5). Each agonist concentration used in this study was ~1 μM (Mackenzie, Word, Casey, and Stull, 1990). Citral (2.2 mM) caused abolition of contractile response induced by both $PGF_{2\alpha}$ and PGE_2. With $PGF_{2\alpha}$, the amplitude, frequency, and AUC of contraction were significant decreased to 59.51±2.50%, 62.80±1.06%, and 66.44±2.25% compared to 100% of the control. With PGE2, the amplitude, frequency, and AUC of contraction were also significant decreased to 42.87±2.70%, 56.50±1.06%, and 57.44±1.30%, respectively ($p < 0.05$, n = 5); compared with the control (100%). The effects were summarized in Table 4.5 and a typical effect is shown in Figure 4.5.

Effects of Camphene on $PGF_{2\alpha}$- and PGE_2-Induced Uterine Contraction

As shown in Figure 4.6 and Table 4.6, spontaneous contraction was increased upon the application of PGs. In the presence of camphene (7.5 mM), the PGs-induced force was slightly decreased in all preparations (n = 5). Camphene also reduced the peak and frequency of PGs-induced contractions. $PGF_{2\alpha}$-induced contractions were significantly decreased myometrial contractions in terms of contraction amplitude, frequency and AUC to 77.32±2.42%, 78.21±2.60%, and 87.29±2.29%, compared with 100% of the control. With PGE_2, the amplitude, frequency, and AUC of contraction were also significant decreased to 78.55±1.32%, 73.91±4.62%, and

84.89±4.93%, respectively ($p < 0.05$, n = 5); compared with the control (100%). The effects were summarized in Table 4.6 and a typical effect is shown in Figure 4.6.

4.4.5 Effects of Citral and Camphene on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction in the Continued Presence of High Ca^{2+}

Effects of Citral on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction in the Continued Presence of High Ca^{2+}

Prostaglandins both, $PGF_{2\alpha}$ and PGE_2, are strong inducers of uterine contraction by promoting external Ca^{2+} into the cell through specific receptors coupled with the calcium channels. Thus, this study was designed to emphasize the importance of external calcium in the PGs-induced rat uterine contraction and to answer whether cital and camphene could alter these contractions by inhibiting the pathways involved external calcium entry. Citral was applied to $PGF_{2\alpha}$-or PGE_2 (1 μM)-induced contraction in the presence of external Ca^{2+}. Force induced by $PGF_{2\alpha}$ or PGE_2 was reduced significantly. The contraction contraction amplitude, frequency, and AUC were reduced (Fig. 4.7 and Table 4.7, n = 5).

Figure 4.7 shown an inhibitory effect of citral on $PGF_{2\alpha}$-and PGE_2-induced contractions, which was significantly recovered. With $PGF_{2\alpha}$, the contraction amplitude, contraction frequency, and AUC were increased to 122.43±3.38, 104.90±2.02, and 116.17±3.35, respectively compared with 100% of the control. With PGE_2, the contraction amplitude, contraction frequency, and AUC were increased to 106.33±2.44, 107.48±2.27, and 124.40±4.90, respectively compared with the control ($p < 0.05$, n = 5).

Effects of Camphene on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction in the Presence of High Ca^{2+}

As shown in Figure 4.8, myometrial contraction induced by PGs is dependent external calcium as camphene or extracellular calcium depletion abolished their effects. It was observed that camphene (7.5 mM) also inhibited PGs-induced force and contractions. The inhibitory effects of camphene on $PGF_{2\alpha}$-induced force and contractions were significantly increased the contraction amplitude, frequency, and AUC to 106.62±1.67, 115.43±2.35, and 108.79±0.61, respectively compared with 100% of the control. With PGE_2, the amplitude, frequency, and AUC of contraction were increased to 104.60±1.80, 109.39±3.68, and 110.63±3.38, respectively compared with control in the presence of high external Ca^{2+}. The data were summarized in Table 4.8 ($p < 0.05$, n = 5).

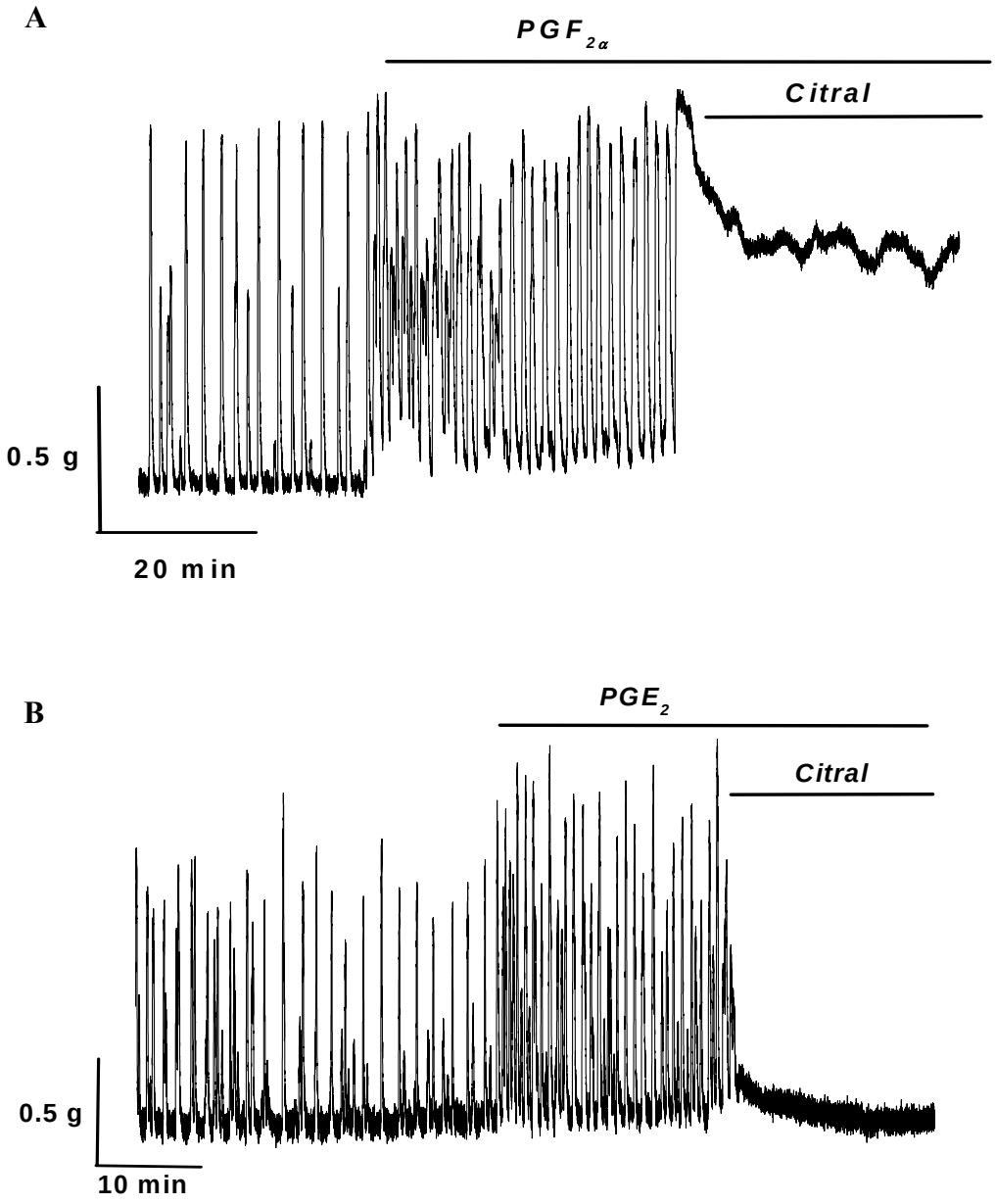

Figure 4.5 The effects of citral on PGs-induced contractions. The effects of 2.2 mM citral on uterine contraction induced by 1 μM $PGF_{2\alpha}$ (A) and 1 μM PGE_2 (B) are shown.

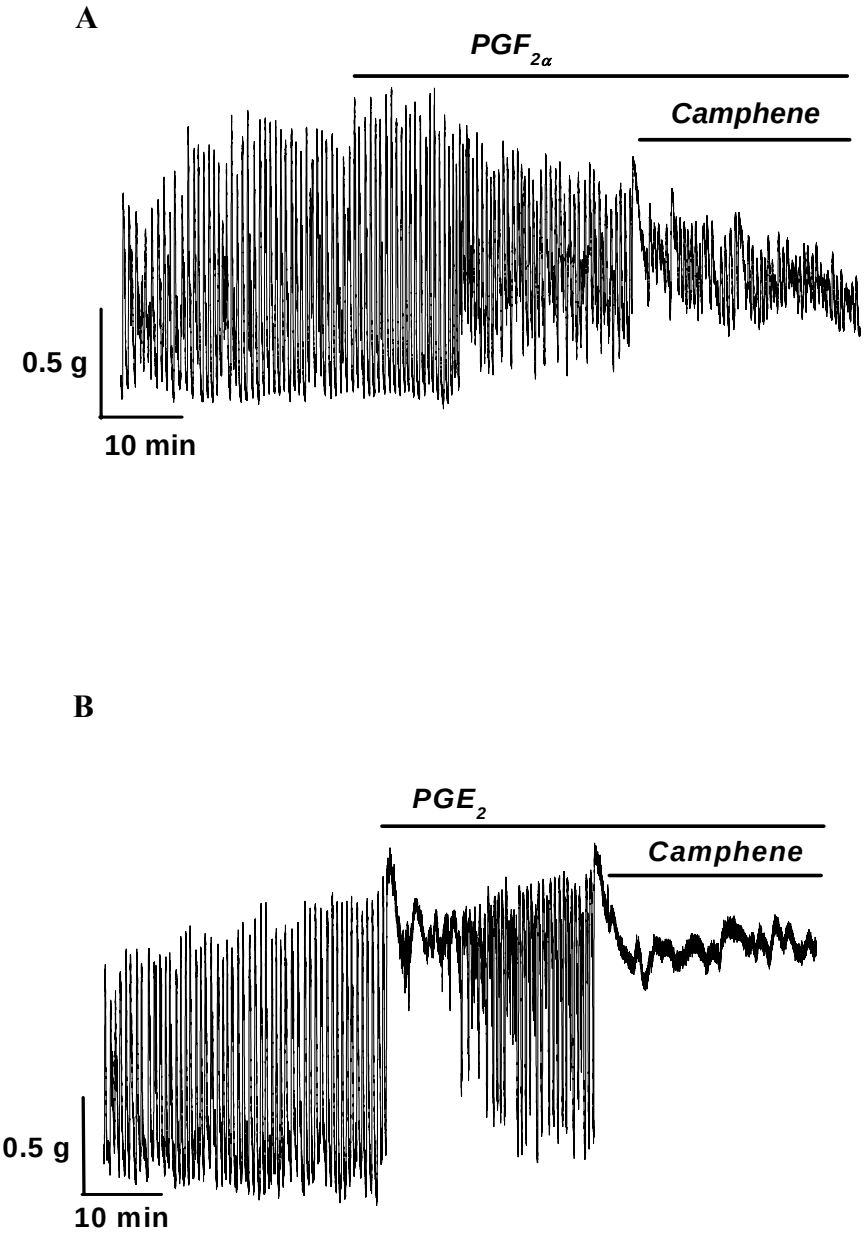

Figure 4.6 The effects of camphene on PGs-induced contractions. The effects of 7.5 mM camphene on uterine contraction induced by 1 μM $PGF_{2\alpha}$ (A) and 1 μM PGE_2 (B) are shown.

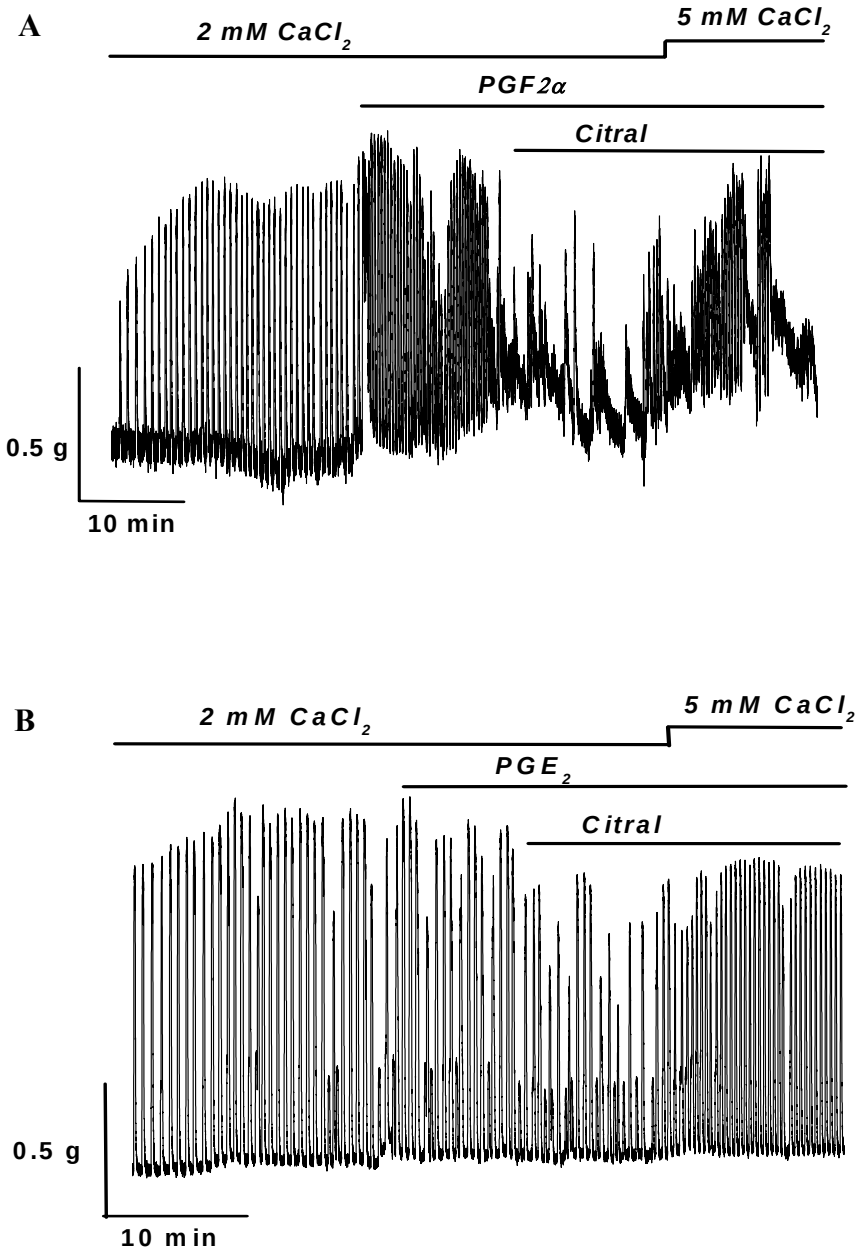

Figure 4.7 The effects of citral on PGs-induced contractions in the present of high Ca^{2+} concentration (5 mM $CaCl_2$). (A) An application of citral on $PGF_{2\alpha}$-induced contractions is shown. (B) An application of citral on PGE_2-induced contractions is shown.

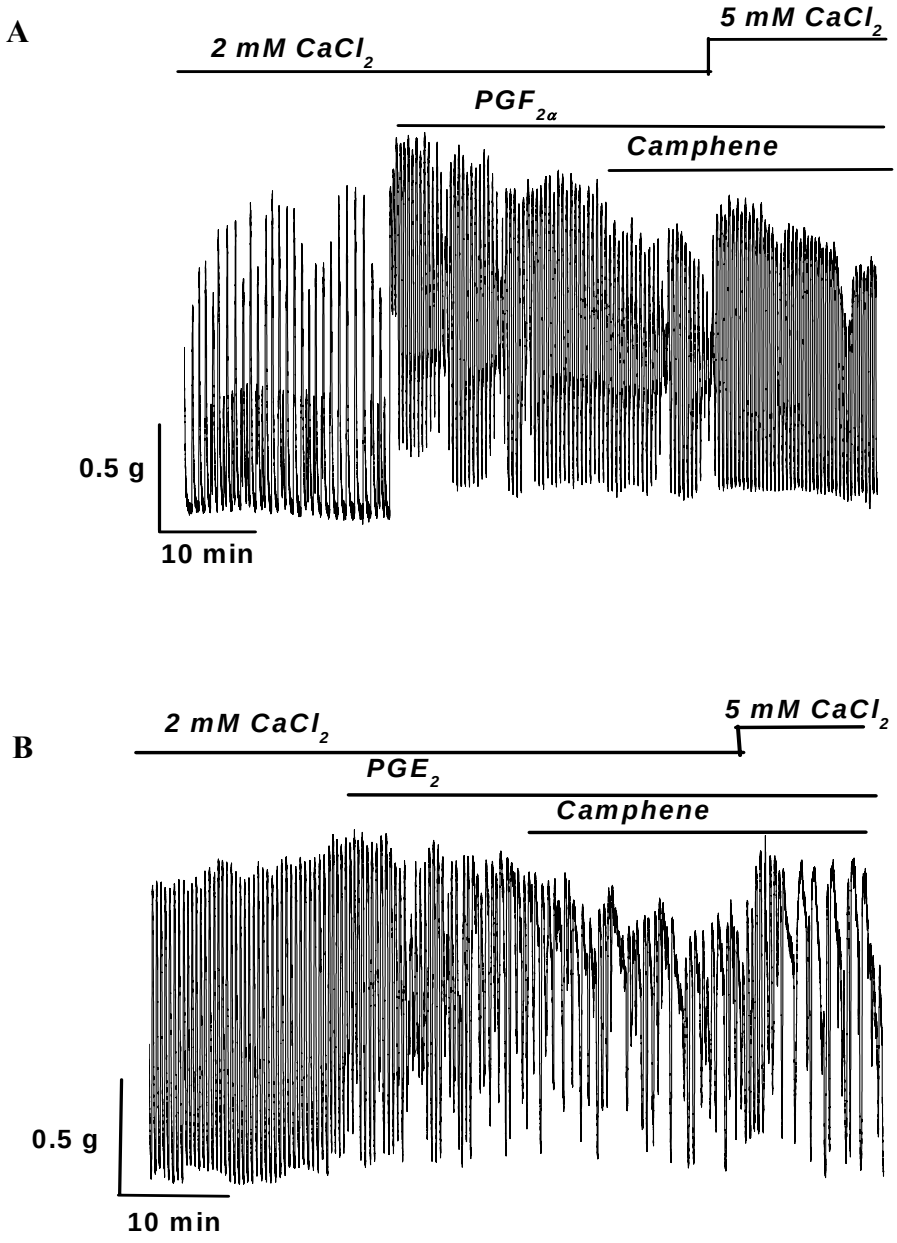

Figure 4.8 The effects of camphene on PGs-induced contractions in the presence of high Ca^{2+} concentration (5 mM $CaCl_2$). (A) An application of camphene on $PGF_{2\alpha}$-induced contractions is shown. (B) An application of camphene on PGE_2-induced contractions is shown.

4.4.6 Effects of Citral and Camphene on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contractions in the Absence of External Ca^{2+}

Effects of Citral on $PGF_{2\alpha}$- and PGE_2 -Induced Uterine Contraction in the Absence of External Ca^{2+}

It has been reported that PGs can induce contraction in the absence of external Ca^{2+} (Luckas et al., 1999). It is interesting to know that whether citral could inhibit such contraction. To do so, $PGF_{2\propto}$ and PGE_2 were added to uterine strip in the absence of external Ca^{2+} using nifedipine. As seen in Figures 4.9 and 4.10 a small amount of force generated upon the application of $PGF_{2\propto}$ and PGE_2. Citral then was added after and in the continued of $PGF_{2\propto}$. As can be seen, force is abolished. This was also the case of citral when it was added before the application of $PGF_{2\propto}$ and PGE_2.

Effects of Camphene on $PGF_{2\alpha}$-and PGE_2-Induced Uterine Contraction in the Absence of External Ca^{2+}

The effects of camphene (7.5 mM) on myometrial contraction induced by $PGF_{2\alpha}$ in the absence of [Ca^{2+}] were investigated. The effects were similarly to the effects of citral and ginger oil. PGs generated a small tonic force in the absence of external Ca^{2+} that slightly developed and maintained as long as PGs were presented (Figs. 4.11 - 4.12). Camphene abolished this contraction, respective of whether it was added before (Figs. 4.11B and 4.12B) and after the PGs application (Figs. 4.11A and 4.12A, n = 5).

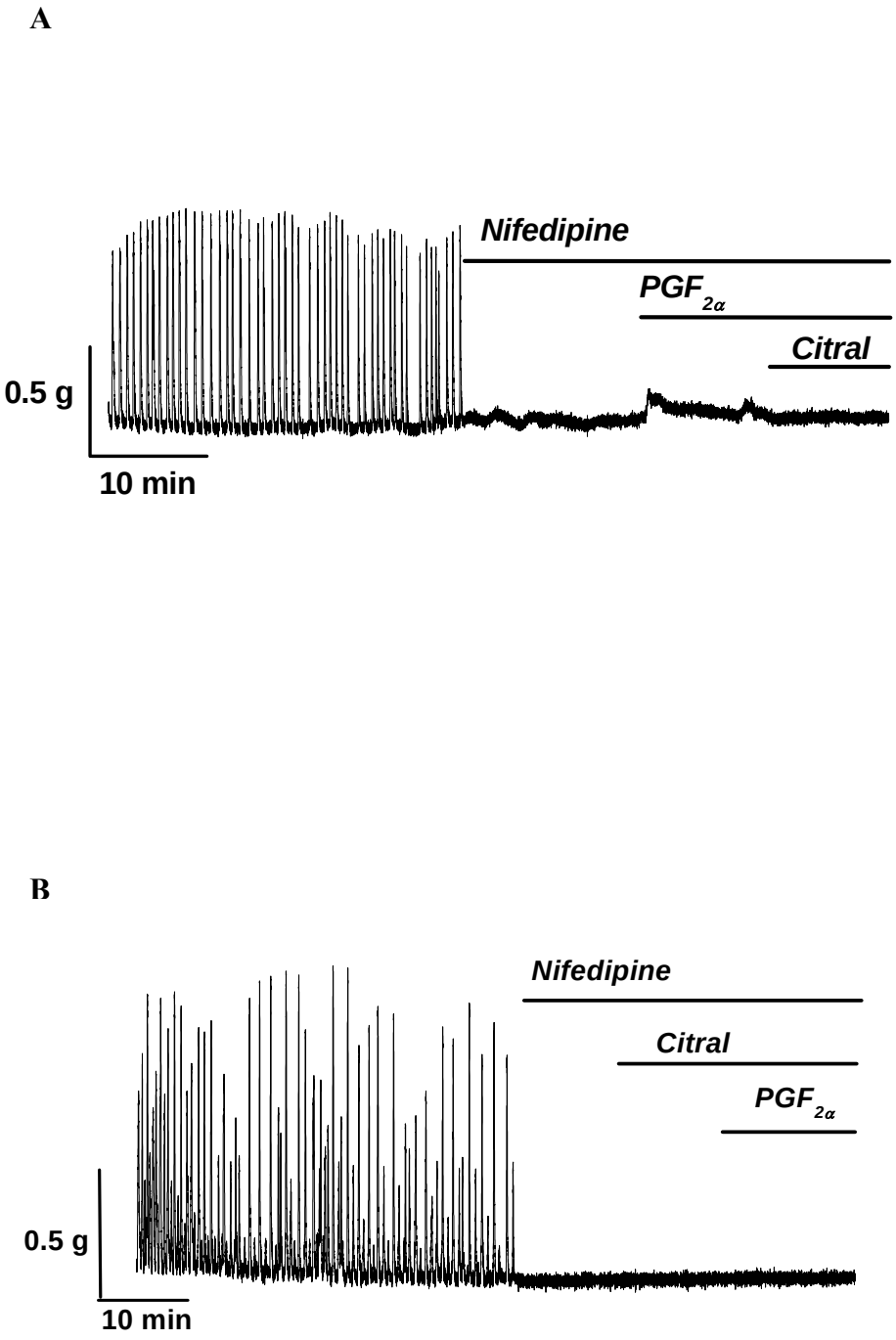

Figure 4.9 The effects of citral in the absence of external Ca^{2+} using 10 μM nifedipine. Citral was added after (A) and before (B) $PGF_{2\alpha}$-induced contraction.

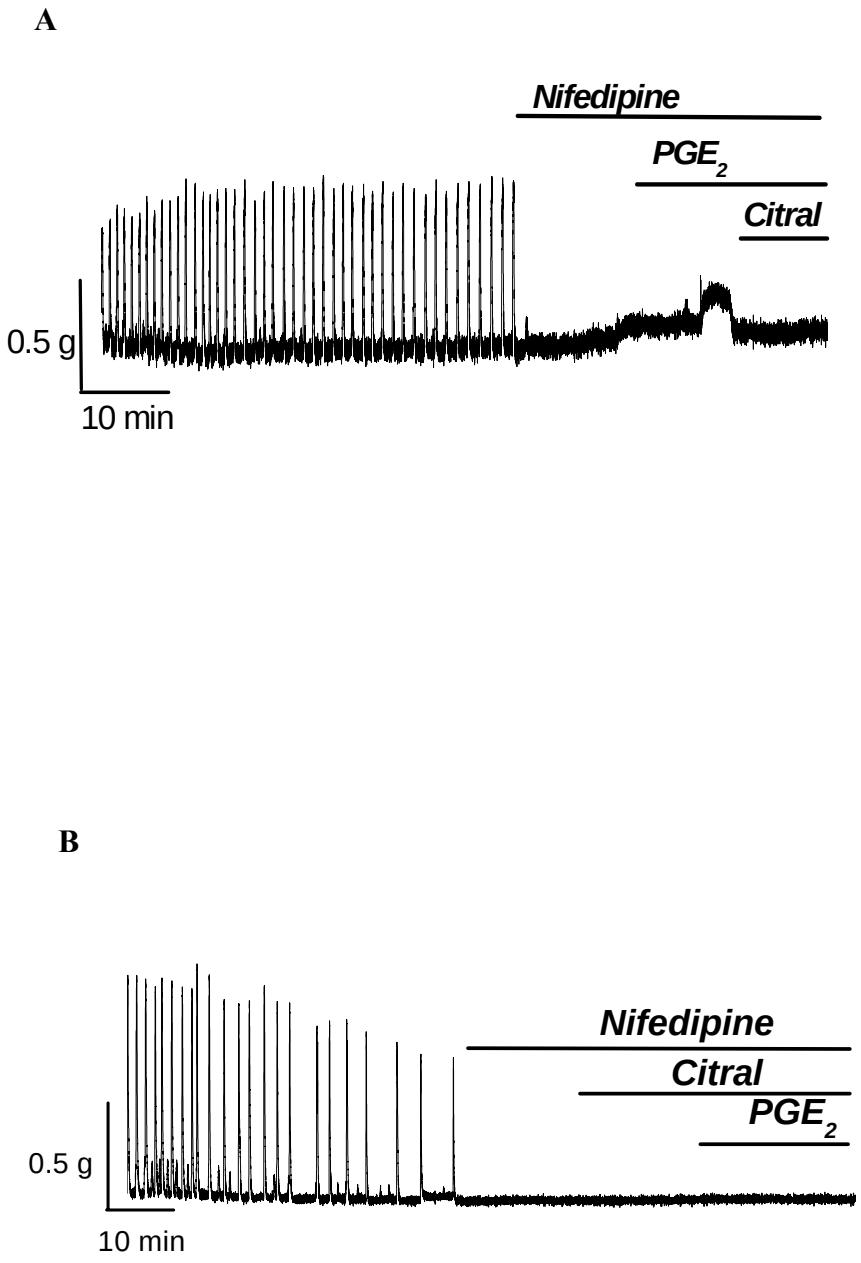

Figure 4.10 The effects of citral in the absence of external Ca^{2+} using 10 μM nifedipine. Citral was added after (A) and before (B) PGE_2-induced contraction.

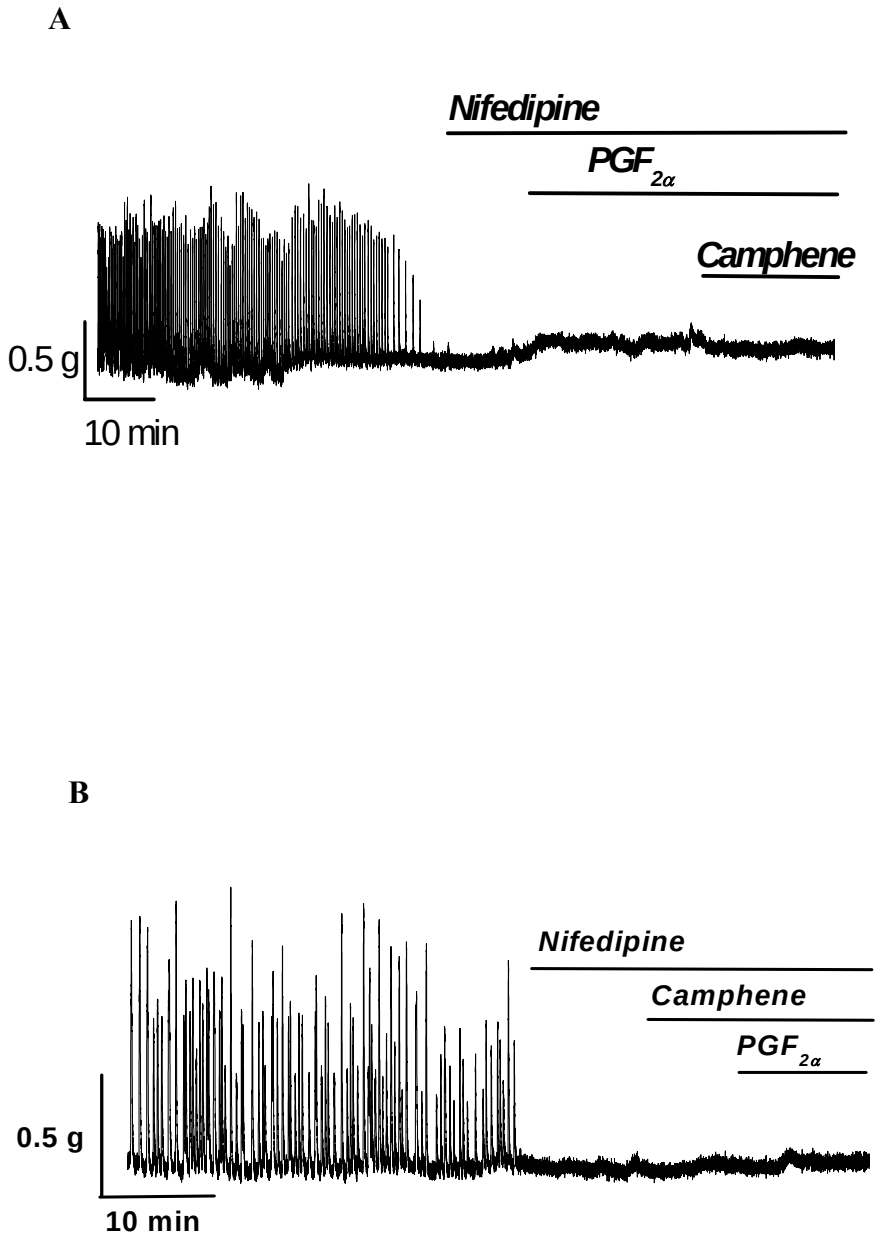

Figure 4.11 The effects of camphene in the absence of external Ca^{2+} using 10 μM nifedipine. Camphene was added after (A) and before (B) $PGF_{2\alpha}$-induced contraction.

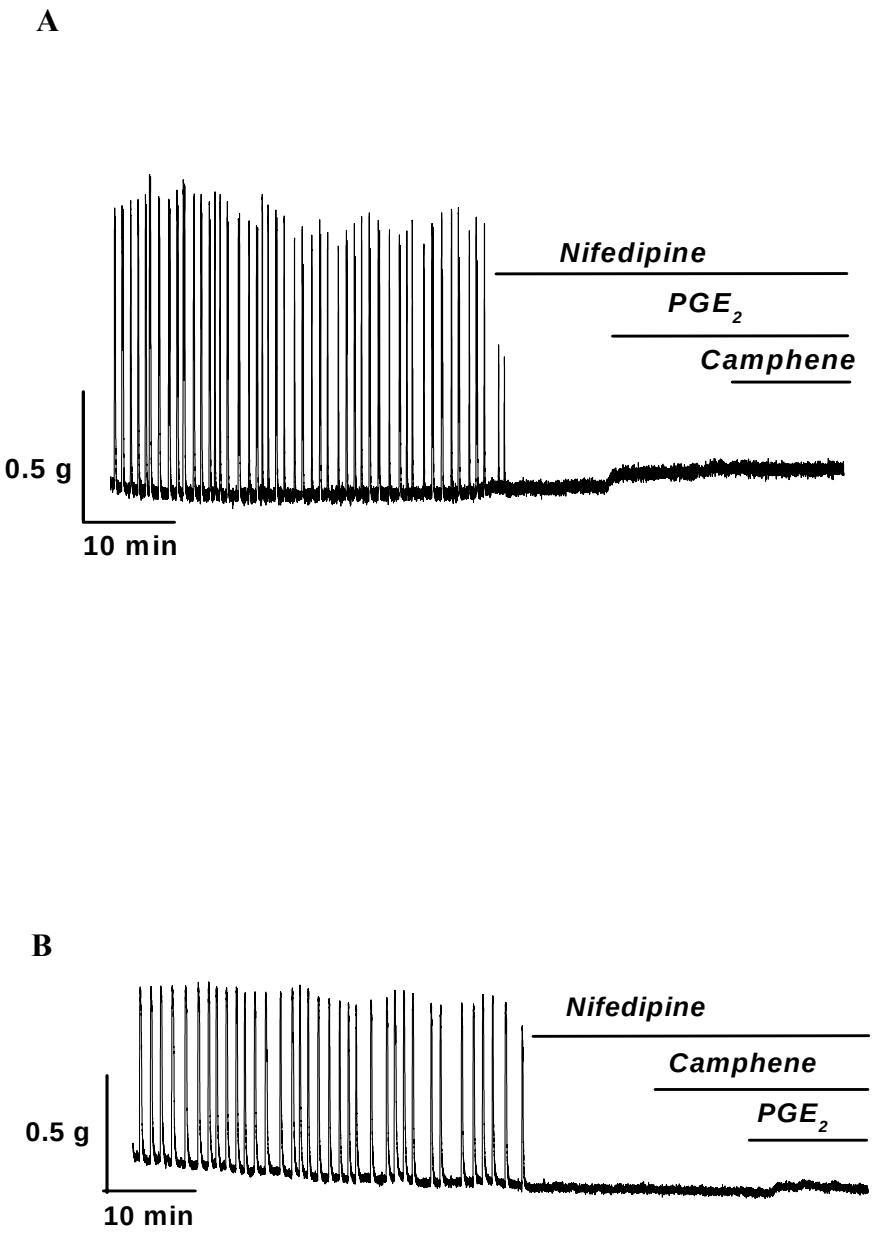

Figure 4.12 The effects of camphene in the absence of external Ca^{2+} using 10 μM nifedipine. Camphene was added after (A) and before PGE_2-induced contraction.

4.4.7 The Effects of BSA on Citral and Camphene

The Effects of BSA on Citral

As mentioned in 3.4.6, the effects of ginger oil on spontaneous contraction could be washed off by 5% BSA. To test whether the effect was the same upon applying citral, BSA was used in this experiment. The inhibitory effects of citral on spontaneous contraction were recorded in the presence of BSA (Fig. 4.13A). The mean values of contraction amplitude, frequency, and AUC were increased to 105.25±.94%, 126.82±0.02%, and 124.24±3.66 %, respectively; compared with the control ($p < 0.05$, n = 5).

The Effects of BSA on Camphene

The strips were exposed to camphene (7.5 mM) after contracting for 30 min. An addition of 5% BSA increased in the mean values of contraction amplitude to 110.65±3.71%, the frequency of phasic contraction to 114.03±2.08%, and AUC to 126.32±4.52%, respectively; compared with the control ($p < 0.05$, Fig. 4.13 B, n = 5).

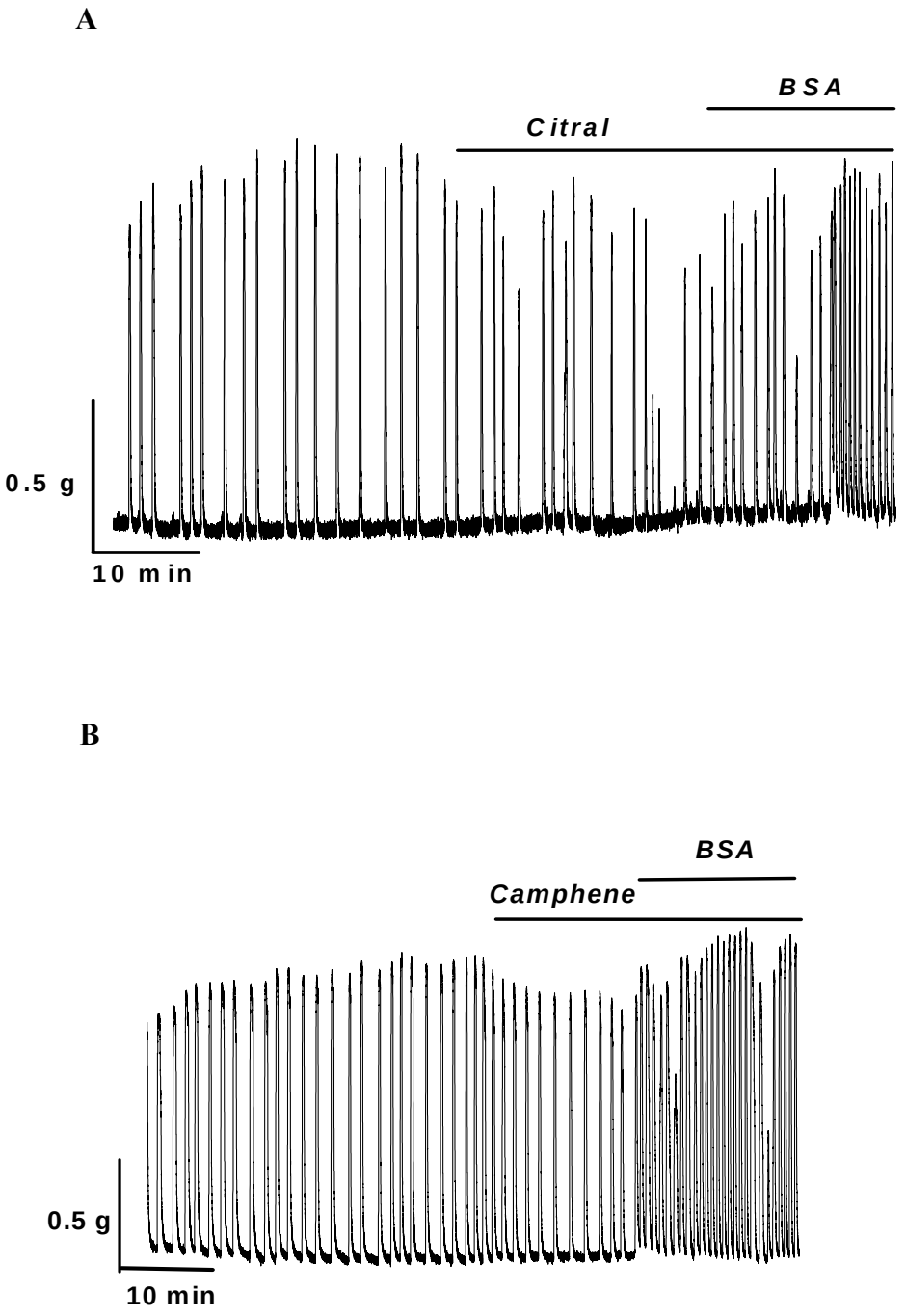

Figure 4.13 The effects of BSA on citral (A) and camphene (B).

4.5 Discussion

Citral and camphene were found as the main constituents of ginger oil as shown in Table 4.1. The inhibitory effects of both compounds on myometrial contraction have not been investigated. Therefore, the aims of this Chapter were to evaluate the effects of citral or camphene on myometrial contractile activities arising spontaneously and by PGs-induced contractions. The results showed that the effects of both citral and camphene were similarly indistinguishable to these of ginger oil; suggesting the effects of ginger oil that found could be due to the effects of citral and camphene.

4.5.1 The Effects of Citral

Citral can inhibit both the contraction arising either spontaneously or by PGs-induced contraction. Its effects were dose dependent. The IC_{50} is at 2.2 mM. The effects found were the same as those observed in ginger oil. Interestingly, its effects were likely to be via inhibition of L-type Ca^{2+} channels. This because, an elevation of external [Ca^{2+}] can reverse the effects of citral. This study showed that both $PGF_{2\alpha}$ and PGE_2 significantly increased spontaneous contractile activity in uterine strips (Carolyn et al., 2002), which are associated with a rise $[Ca^{2+}]_i$ by external Ca^{2+} entry and intracellular Ca^{2+} stores. They also induced a tonic contraction in the absence of external Ca^{2+}. Interestingly, citral inhibited the contraction occurred in both the presence and absence of external Ca^{2+}. This is similar to the effect of ginger oil as shown in Chapter III.

Thus, PGs involved Ca^{2+} influx through L-type Ca^{2+} channels (Coleman et al., 2000) were also blocked by citral on their activities (Figs. 4.5 and 4.6). However an

arising of external Ca^{2+} (5 mM $CaCl_2$) or PGs-stimulated myometrial contraction could be reappeared to force and contract again in the presence of citral at IC_{50} concentration (Figs. 4.4 and 4.7). As can be seen in Figures 4.9 and 4.10, there have no contraction when external Ca^{2+} entry was blocked by nifedipine (10 μM) and abolished PGs-induced force in the presence of citral.

4.5.2 The Effects of Camphene

Another known compound of ginger constituent, camphene, was also studied. This is the first study of its inhibitory effects on myometrial contraction *in vitro.* No other known compounds such as zingeberene, or curcumene, are commercially available, and so could not be investigated.

These expreiments were designed to investegate and to compare the inhibitory effects of camphene on spontaneous or PGs-induced contraction with the effects of citral and ginger oil. The results indicated that (0.5 to 1.5 mM) camphene had no significant different of contraction frequency, amplitude and AUC when compared to control (100%). These findings suggest that its effective seem to be weaker compared with the effects of citral and ginger oil. However, at the same concentration, the inhibitory effects of citral is the strongest as it can inhibit both spontaneous and PGs-induced myometrial contractions. Also, camphene at IC_{50} was found to inhibit Ca^{2+} influx in myometrial strips (Fig. 4.4B). And there was no response contraction when external Ca^{2+} was blocked by nefedipine (10 μM) in the presence of camphene (Figs. 4.11-12). However, the amplitude contraction, frequency, and AUC were increased higher than spontanneous contraction once adding BSA (Fig. 4.13B). Those experiments conclude that its effects were in accordance with the inhibition of volatile

oil of *Teucrium polium in vitro* of isolated rabbit intestine. Thus, the effect was likely to be due to camphene inhibits Ca^{2+} influx through the potential dependent Ca^{2+} channels (Agel and Gharibeh, 1990).

In conclusion the present study shown that two known commercial available compounds may be to the active compounds of ginger oil. Particularly, the results indicated that their inhibitory effects may act on Ca^{2+} dependent pathways. Based on the present finding, citral showed the higher potent inhibition on external Ca^{2+} required for myometrial contractile activities. In addition, it is clearly demonstrated that the inhibitory effects of both citral and camphene may be involved in the pathway of non-Ca CaM MLCK.

4.6 References

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CHAPTER V

EFFECTS OF GINGER OIL, CITRAL, AND CAMPHENE ON INFLAMMATORY MEDIATED PATHWAYS IN RAT MYOMETRIUM

5.1 Abstract

Several studies have been reported that cyclooxygenase (COX) catalysis is the first step of arachidonic acid (AA) mechanisms of PGs synthesis. An increased expression of COX-2 enzyme participates to a dramatic PG rates rising in inflammation tissues. Previous studies suggested that an anti-inflammatory process was depressed by ginger constituents. Therefore, the aims of this chapter were to examine whether the inhibitory effects of ginger oil, citral, and camphene on spontaneous contraction in non-pregnant rat myometrium are via COX-2 or via phosphodiesterase (PDE) pathways. There were three main aims; 1) to assess the inhibitory effects on AA-induced myometrial contraction 2) to measure PGE_2 and cAMP contents and 3) to determine the inhibitory effects contribution of indomethacin in the myometrial contraction. The rat myometrial strips were prepared. The effects of exposing the strips to AA, indomethacin and aminophylline were investigated. Exposure of AA increased the amplitude, frequency, and AUC of spontaneous contraction. AA-induced contraction was inhibited in the presence of ginger oil and its pure compounds. As with ginger oil, indomethacin inhibited the amplitude of spontaneous contraction, but did not affect the frequency and AUC of

contractions. Assay of COX-2 enzyme showed no effect of its activities in this condition. Aminophylline (1 nM), ginger oil, citral, and camphene remarkably inhibited PDE enzyme but did not cause an accumulation of cAMP content. These results indicate that the inhibitory effects on spontaneous contraction of ginger oil and others did not occur via inflammatory process and that this neither involved with COX-2 pathway nor cAMP production.

5.2 Introduction

Inflammation is associated with a large range of mediators that initiates inflammatory response for AA release by cell biosynthesis of prostaglandins (PGs) via the cyclooxygenase (COX) pathway. The COX isoenzymes, COX-1 and COX-2 (His et al., 1994; O'Banion et al., 1992) pathway produces PGs (Simmons et al., 2004), prostacyclins and thromboxanes. PGs are important biological mediators essential for physiological functions in the body that control inflammatory responses, pain, and fever (De Witt, 1991). An increase production of prostaglandins during an inflammatory response in achieved by induction of COX-2 (Ramsay et al., 2003). Activation of COX results in the synthesis of a wide spectrum of prostaglandins (Kuby, 1997) the critical mediators of the inflammatory process, with various activities leading to increased vascular permeability, increased vasodilation (Kuby, 1997). In addition, the role of PG activities are involved in various processes associated with reproduction (Vane et al., 1988), ovulation (Lemaire and Marsh, 1975), fertilization (Lim et al., 1997), implantation (Chakraborty et al., 1996), maintenance of normal pregnancy (Kennedy, 1977; Trautman et al., 1996), and induction of labor (Hoffman, 1978). COX converts AA to PGG_2 through oxygenase

activity, and this unstable product is reduced to PGH_2 through peroxidase activity (Smith and Marnett, 1991).

A second messenger role of AA in signal transduction is widely accepted (Axelrod, 1990; Khan et al., 1995). As the evidence of an experiment suggested that changes in intracellular AA concentration could cause pathological effects such as ischemia and neurotrauma (Bazan et al., 1995; Oe et al., 1994; Katsura et al., 1993).

However, the various kinds of drugs had been developed to inhibit these enzyme (COX-1, COX-2), non-steroidal anti-inflammatory drugs (NSAIDs), are devided into two groups: COX-1/-2 non-selective inhibitors (Meade et al., 1993; O'Neill et al., 1994; Cromlish et al., 1994) and COX-2 selective inhibitors (Copeland et al., 1994; Seibert et al., 1994; Futaki et al., 1994). COX-2-selective-NSAIDs have been used to inhibit pain, fever, inflammation and intestinal neoplasia in mice and human (Mann and DuBios, 2004) by inhibiting the COX pathway. COX inhibitors, nonstelective drugs or COX-2 NSAIDs such as piroxicam, aspirin, diclofenac, particularly indomethacin, are among the most potent tocolytic agents available (Vermillion and Landen, 2001). However, like other classes of drugs for tocolytic, their efficacy is limited. These effects have been focused on finding lead anti-inflammatory compounds from natural products because of NSAIDs side effects. Determining for avoiding the NSAIDs side effects or problem in therapeutics that damage with protracted use include dizziness, headache, nausea, vomiting and diarrhea gastrointestinal tract (Cahill, 1986; Bjarnason et al., 1986). The experiments are therefore design to suppress inflammation without their side effects of the present range of NSAIDs.

Several studies have indicated that compounds found in ginger are effective in relief of symptoms from chronic inflammatory diseases and rheumatoid arthritis (Srivastana and Mustafa, 1992). On the one hand, it was *in vitro* observed that gingerdione inhibited the production of 5-HETE (leucotrienes) and PGE_2. Additionally, it way proved that shogaol inhibited the production of leucotrienes. Gingerol, as well as dehydroparadol, favored the COX inhibition (Srivastava, 1984; Kiuchiet et al., 1992; Mustafa et al., 1993; Tjendraputra et al., 2001). It was demonstrated that ginger was a potent inhibitor of the prostaglandin synthesis, even stronger than indomatacin (Mustafa et al., 1993). On the other hand, it was *in vivo* observed that ginger acted by inhibiting the prostaglandin synthesis and the production of free radical during metabolism of the AA (Mustafa et al., 1993). Recently study, ginger is one of most valuable effects its ability to reduce and relieve inflammatory pain as effectively as aspirin, ibupofen, and other NSAIDs, without their adverse side effects. Ginger does this by partial blocking COX-2 enzymes, which are necessary for inflammation. Moreover, an inhibitor of PG synthesis, ginger root has been used for thousand of years for its anti-inflammatory properties (Srivastava and Mustafa, 1989; Taymor et al., 1964). In additional medicine, ginger has been used to treat many inflammatory conditions and associated pain. Furthermore, ginger constituents, (8)-paradol and (8)-shogaol, as well as to synthetic analogues, 3-hydroxy-1(-4-hydroxy-3-methoxyphenyl), decane and 5-hydroxy-1-(4-hydroxy-1-3-methoxyphenyl) dedecane**,** showed strong inhibitory effects on COX-2 enzyme activity (Tjendraputra et al., 2001).

However, the mechanisms of AA action on smooth muscle remain to be identified. Adding AA leading to contraction activated from the intracellular side of

the plasma membrane, in accordance with other researchs, different protein kinase C isoforms can be activated by AA (Lester et al., 1991; Khan et al., 1995). AA stimulated protein kinase C binding to actin filaments (Prekeris et al., 1996) and related lipid messengers were reported to sensitize the contractile smooth muscle apparatus to basal Ca^{2+} concentrations (Gong et al., 1992). Moreover, Filipeanu et al. (1998) showed that different mechanisms are involved in the contractile effect of intracellular and in the effect of extracellular AA in rat aorta. Previous studies have found that it also functions by prostaglandin independent mechanisms, such as inhibition of nitric oxide synthase and phosphodiesterase 4 (Bruch et al., 1983; Bevilacqua and Magni, 1993) related cAMP. In addition, the study on uterine smooth muscle suggested that papaverine inhibited smooth muscle contraction mainly by inhibition of mitochondrial respiration and did not increase in cAMP level (Shimizu et al., 2000). In smooth muscle, cAMP modulation has been postulated to involve stimulation of Ca^{2+} and Na^{+} pumps (Scheid et al., 1979; Bulbring and den Hertog, 1980), partial inhibition of potential-operated Ca^{2+} channels (Meisheri and van Breemen, 1982). It also decreased in the affinity of myosin light chain kinase for the Ca-calmodulin complex (Adelstein et al., 1978, 1982).

Nevertheless, there have been a few reports indicating that PDE3, 4, and 5 inhibitors caused relaxation in canine colonic smooth muscle, and that PDE3 and 4 inhibitors decreased the hydrolysis of cAMP, and PDE5 inhibitor decreased the hydrolysis of cGMP (Barnette et al., 1993). At the cellular level, the intensity and the duration of the intracellular cAMP and cGMP signals are partly regulated by the PDE enzymes whose functions are to degrade cyclic nucleotides into their inactive metabolites. PDE4 is selsctive for cAMP hydrolysis did, and it has been widely

evidenced that the elevation of cAMP levels by PDE4 inhibition relaxes various types of smooth muscle fibers (Conti et al., 1995). The role of the cAMP-specific PDE 4 family has seldom been investigated (Ahn et al., 1992). However, the relaxant effects of pregnant rat uteri were not mediated by cAMP when the arising of external Ca^{2+} concentration by using aminophylline, an inhibitor of cAMP-PDE enzyme (Apaydin et al., 1998). Additionally, with regard to uterine smooth muscle of non-pregnant rats, the cAMP-independent mechanisms remain unclear**.**

The aims of this Chapter were to determine whether the inhibitory effect of ginger oil and its active compounds involved in inflammatoty process. AA was used to induce contraction. The effects of ginger oil and its pure compounds together with aminophylline, the non-specific PDE enzyme inhibitor, and indomethacin, the COX-2 inhibitor, on uterine contraction were studied. The production of cAMP was also investigated.

5.3 Materials and Methods

5.3.1 Chemicals and Physiological Solution

All chemicals were purchased from Sigma® unless state otherwise. Agonists/antagonists for the investigation of inflammatory pathways were 95% citral, 95% camphene, indomethacin, aminophylline and AA, cAMP EIA kit (cAMP Biotrak EIA system, Amersham Pharmacia Biotech, UK) and PGE_2 assay kit. Ginger oil and its pure compounds were dissolved by hexane. The final concentration of hexane in any dilution was less than 0.15%. DMSO was used for PGE_2 assay.

5.3.2 Myometrial Tissue Preparations

Tissue preparations are essentially the same as those described in Chapter II. Non-pregnant Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, SUT, Thailand. Myometial tissue preparations were dissected and provided for tension measurements as described in 2.2.2.

5.3.3 Measurements of Tension

The uterine strips were mounted vertically under resting tension of 1 g in a single chamber (25 ml) tissue bath connected to a force transducer (as described in 2.3). Tissues were allowed to contract spontaneously. After an equilibrium period for 30 min, either AA or indomethacin was applied. The data were recorded as the electrical signal from the transducer and converted to a digital signal on a computer using Chart software.

5.3.4 Anti-Inflammatory RIA Assay

5.3.4.1 Cell Culture and Treatment

Immortalized mouse PGH-1 and PGH-2 null cells at the concentration (1×10^5 cells/ml) of Dubelcco's Modified Eagle Medium (DMEM) high glucose supplemented with hygromyein B (200 μg/ml), non essential amino acid (0.1 mM), L-glutamine (50 mg/l), ascorbic acid (0.05 mg/ml) and 10% fetal calf serum (FCS) were seeded into 96-well flat bottom tissue culture plates (83 μl/well). Cells were incubated

37°C in humidified incubator with 5% CO_2 for 7.2 hr. The cells were then washed with DMEM medium without FCS and pre-incubated for 30 min with 83 µl of serum-free DMEM medium containing vehicle or drugs. Following the pre-incubation period, the medium was removed and cells were immediately treated with serum-free medium containing vehicle or drugs and AA (20 µM) or A 23187 (2 µM) for 30 min. Culture supernatants were then collected from wells and analyzed for PGE_2 concentrations by radioimmunoassay (RIA).

5.3.4.2 PGE_2 Measurement

The RIA method used for measuring PGE_2 concentrations in the culture supernatant is based on the competition between PGE_2 in samples and ^3H labeled PGE_2 for anti-PGE_2 antibody binding sites. The assay was performed on ice as following. The supernatant was diluted with DMEM (1:10) for blank and zero % binding and added approximately 1.5 ml into 50 µl micro-centrifuge tube. Then, 50 µl of anti-PGE_2 antibody in RIA buffer (0.1 mM phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.1% sodium azide and 0.1% gelatin) was added to every tube accept for the blanks, in which 50 µl RIA buffer was added. Subsequently, 50 µl of ^3H-PGE_2 (1.12 µci/ml), was added to each tube and vortexed briefly, then incubated overnight at 4°C. Then, 100 µl at 2% charcoal-dextran suspension in RIA buffer was added to each tube. After 15 min incubation on ice, the tubes were centrifuged at 3,800 rpm (1,500 g) at 4°C for 10 min. Supernatants were then transferred to new 1.5 ml microcentrifuge tubes containing liquid scintillation cocktail, vortexed, and counted for radioactivity. The resulting radioactive counts were used to calculate a

certain percentage of ^{3}H-PGE_2, which were then used for the estimation of PGE_2 concentrations from standard curves.

5.3.5 cAMP EIA Assay

In the muscle strips, cAMP content were measured by enzyme immunoassay (EIA) as reported previously (Shimizu et al., 200; Kaneda et al., 2005). The uterine strips were incubated with 5 mM $CaCl_2$ for 10 min then with aminophylline, ginger oil and its pure compounds (citral and camphene) for 30 min at 37°C. After incubation with various treatments, the strips were rapidly frozen in liquid nitrogen and stored at -80°C until homogenized in 6% trichloroacetic acid (0.4 ml). The homogenate was centrifuged at 3000×g for 15 min and the supernatant was washed with 1.5 ml of water-saturated diethylether four times; the cAMP contents were assayed by using EIA kit (Amersham Pharmcia Biotech, Little Chalfont, UK). cAMP contents were expressed as fmol/g wet weight.

5.3.6 Data Analysis

5.3.6.1 Statistic Analysis for Measurements of Tension

All data were then presented as mean ± S.E.M. and "*n*" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests or ANOVA and P values < 0.05 taken to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100%).

5.3.6.2 Statistic Analysis of PGE_2 Contents

The paired *t*-test procedure was used to determine the differences in the PGE_2 levels between control samples of wild-type. COX-2 cells and among control samples and samples from cytokine-treated cells were collected. Differences were considered significant if $p < 0.05$.

5.3.6.3 The cAMP EIA Assay

Data were calculated as the average optical density (OD) for each set of replicate wells. The percent bound for each standard and sample (see below) was calculated.

$$\%B/B_0 = \frac{\text{(standard or sample OD-NSB OD)}}{\text{(zero standard OD-NSB OD)}} \times 100$$

5.4 Results

5.4.1 Measurements of PGE_2 Contents (COX-2 Assay)

PGE_2 biosynthesis is regulated by successive metabolic steps involving the phospholipase A_2 mediating release of AA and its conversion to PGE_2 by COX, hydroperoxyidase and isomerase (Coetzi et al., 1995). Following the preincubation period, the medium containing vehicle or drugs and AA (20 μM) was prepared for 30 min. Samples were then collected to the RIA method (Kirtikara et al., 1998; Kirtikara et al., 2001) used for measuring PGE_2 contents were measured by RIA method in the cell culture based on the competition between PGE_2 in the samples and ^3H label PGE_2 for antibody binding sites. Anti-inflammation of ginger oil and its pure compounds was tested by RIA to compare with control (aspirin 0.9 – 1.0×10^{-5} g/ml, the COX-2

inhibitor). 10^{-5} g/ml such as ginger oil, 95% citral, and 95% camphene were inactive for anti-COX-2 (the inhibition less than 50%, containing DMSO vehicle, Table 5.1).

Table 5.1 Effects of ginger oil, citral, and camphene on COX-1, COX-2 pathways.

	Anti-COX-2		Anti-COX-1	
Samples	Activity	IC_{50}	Activity	IC_{50}
Citral (95%)	Inactive	-	-	-
Ginger oil	Inactive	-	-	-
Camphene (95%)	Inactive	-	-	-

IC_{50} of positive control: Aspirin = 0.9 - 10×10^{-5} g/ml (COX-2), and 0.4 – 0.5×10^{-5} g/ml (COX-1), maximum final concentration of tested samples: 10^{-5} g/ml, negative control: 0.1% DMSO

Interpretation:	% inhibition	Activity
	< 50%	Inactive
	≥ 50%	Active (IC_{50})

5.4.2 Effects of Ginger Oil, Citral, Camphene, and AA on the Spontaneous Contraction

Spontaneous contraction of rat myometrial tissues was stimulated and increased after equilibrium with AA (20 μM) for 30 min. It was found that AA induced significantly increase in the mean amplitude, frequency and AUC of contraction up to 134.61±1.65, 114.52±5.02, 134.61±1.65, and 133.024±3.91%, respectively ($p < 0.05$, n=10), compare with the control (Figs. 5.1 and 5.3A).

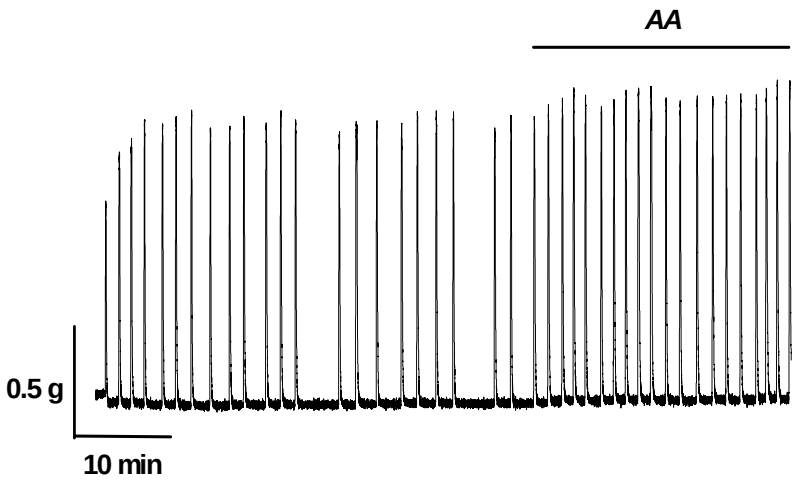

Figure 5.1 The effects of AA (20 μM) on spontaneous contraction.

5.4.3 Effects of Ginger Oil and Its Pure Compounds on the Presence of AA-Induced Rat Myometrial Contraction

Under control conditions, AA (20 μM) was added to spontaneously contracting uterus and its effect was compared to the control. As shown in Figures 5.2, 5.3B and Table 5.1, (n = 5) ginger oil, 95% citral (2.2 mM), and 95% camphene (7.5 mM) decreased AA-induced contraction. Ginger oil (50 μl/100 ml) significantly reduced the contractile activity induced by AA (20 μM) in terms of mean amplitude, frequency and AUC (76.25±7.70, 75.56±4.78, and 59.19±8.39%, respectively (n = 5). The effects were weaker than that of citral (60.95±4.75, 43.83±2.55, and 54.37±1.23%, respectively), but stronger than that of camphene (90.46±7.19, 75.17±7.18, and 91.63±2.29%, respectively (n = 5).

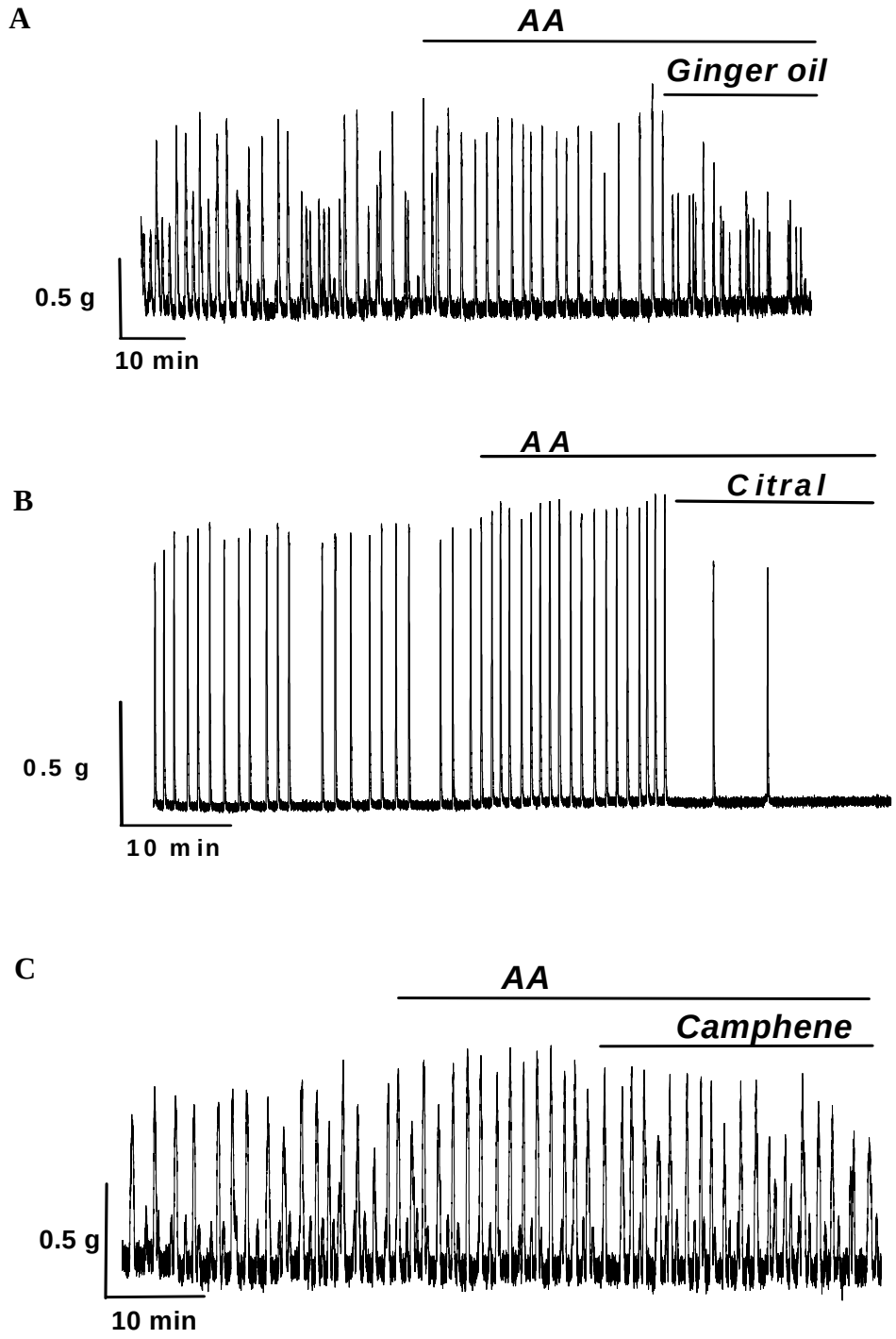

Figure 5.2 The effects of ginger oil (A, 50 µl/100 ml), 95% citral (B, 2.2 mM), and 95% camphene (C, 7.5 mM) on spontaneous contraction in the presence of AA (20 µM).

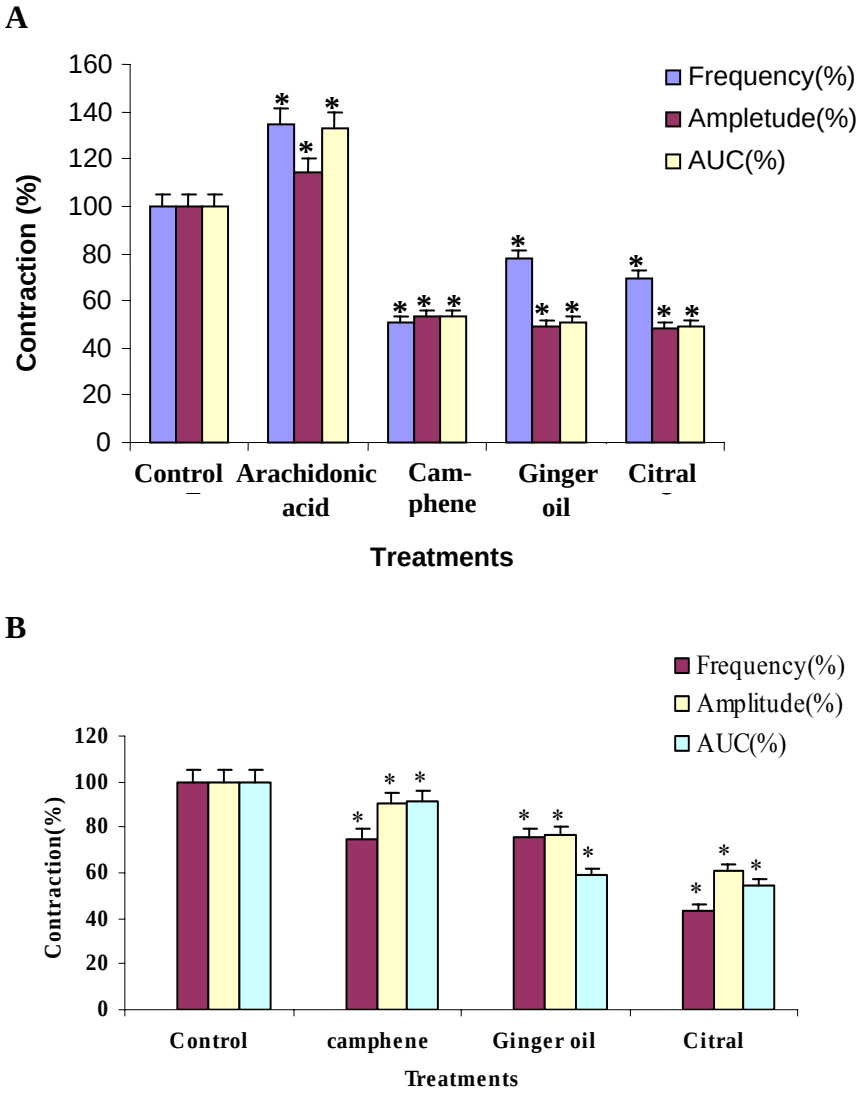

Figure 5.3 The effects of AA (20 µM), ginger oil (50 µl/100 ml), 95% citral (2.2 mM), and 95% camphene (7.5 mM) on spontaneous contraction. (A), (B) Effect of ginger oil (50 µl/100 ml), 95% citral (2.2 mM), and 95% camphene (7.5 mM) on myometrial contraction in the presence of AA (20 µM). Vertical bar indicates the S.E.M.*: Significant difference from the respective control with $p < 0.05$.

5.4.4 Effects of Indomethacin on Spontaneous Contraction

As shown in 5.4.3, ginger oil, citral, and camphene could inhibit AA-induced contraction. To test whether these effects resembled to the inhibition of AA, indomethacin was applied to spontaneous contraction (Figs. 5.4 and 5.6A) and its effect compared with those of ginger oil, citral, and camphene. Adding indomethacin alone (20 μM, Fig. 5.4) to spontaneous contractile activity of rat myometrail tissues for 30 min significant decreased the mean amplitude, frequency, and AUC of contraction to 78.33±5.70, 71.87±3.90, and 72.94±4.87 %, respectively ($p < 0.05$, n = 5), compared with the control (100%).

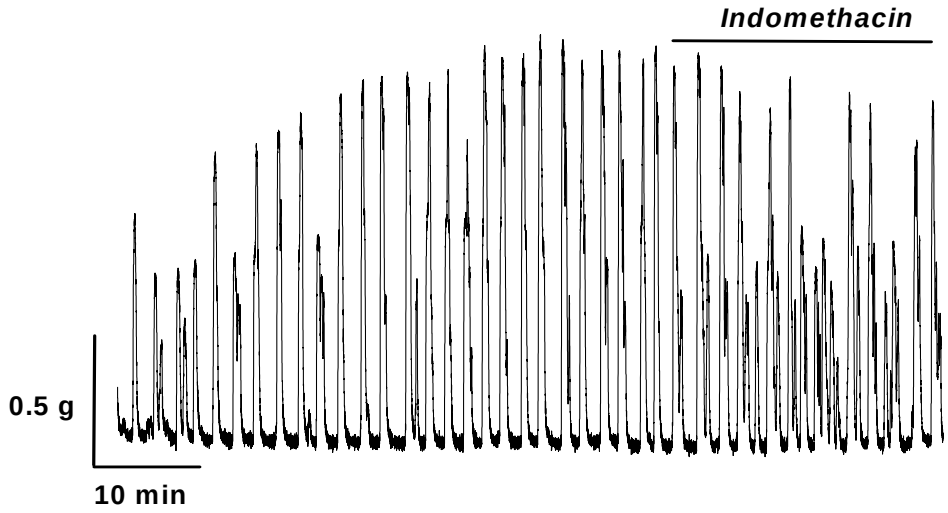

Figure 5.4 The effects of indomethacin (A, 20 μM) on myometrial contraction.

The effects of combination of indomethacin and ginger oil, citral, and camphene were also tested. Indomethacin combined with ginger oil (50 µl/100 ml) significant decreased mean amplitude, frequency, and AUC of contraction to 60.92±3.65, 36.51±8.87, and 61.71±7.14% ($p < 0.05$, n = 5), respectively. As with ginger oil, indomethacin combined with 95% citral (2.2 mM) significant decreased in mean amplitude, frequency, and AUC of contraction to 58.37±8.55, 63.51±6.12, and 50.78±4.00% ($p < 0.05$, n = 5), respectively. Again, indomethacin combined with 95% camphene (2.2 mM) significant decreased mean amplitude, frequency, and AUC of contraction to 68.78±8.47, 50.04±5.16, and 64.08±3.39% ($p < 0.05$, n = 5), respectively. The data were summarized in Table 5.2 and a typical effect is shown in Figures 5.5 and 5.6B.

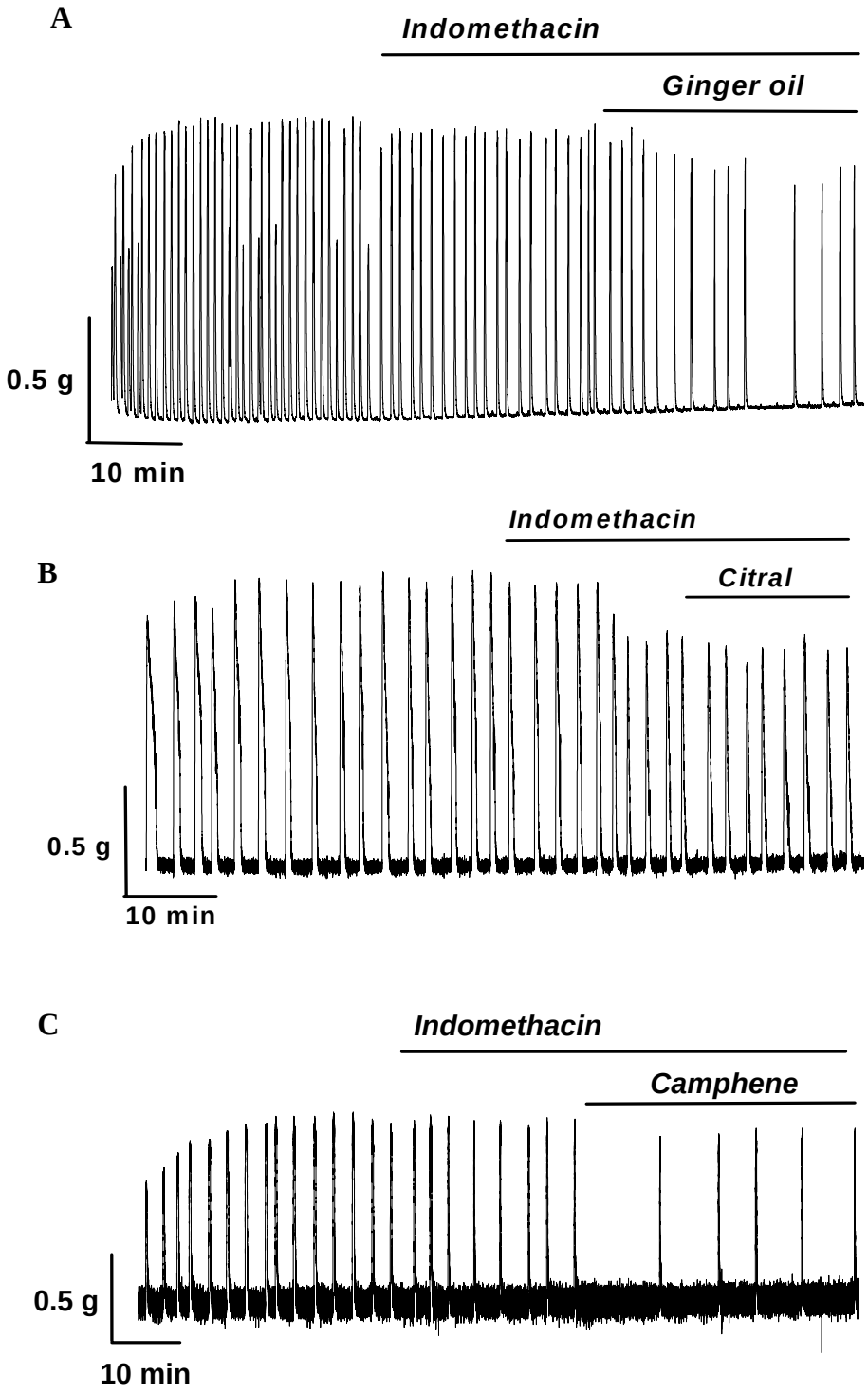

Figure 5.5 The effects of ginger oil (A, 50 µl/100 ml), 95% citral (B, 2.2 mM), and 95% camphene (C, 7.5 mM) on spontaneous contraction in the presence of indomethacin (20 µM).

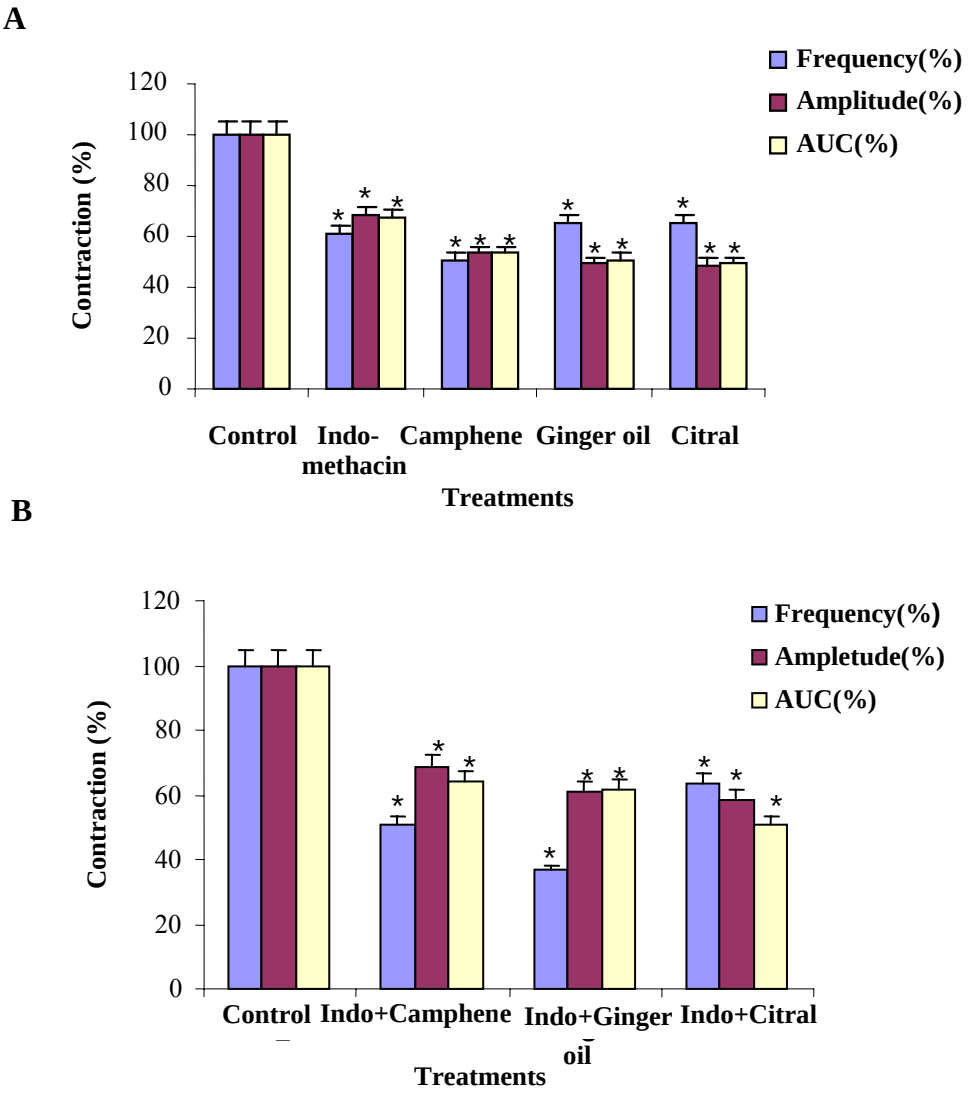

Figure 5.6 The effects of indomethacin (20 µM), ginger oil 50 µl/100 ml, 95% citral (2.2 mM), 95% camphene (7.5 mM) on spontaneous contraction (A), (B) effects of ginger oil, citral, and camphene on myometrial contraction in the presence of indomethacin (Indo-, 20µM). Vertical bar indicates the S.E.M. *: Significant difference from the respective control with $p < 0.05$.

5.4.5 Measurement of cAMP contents

The cyclic AMP content on rat myometrial strips were measured after pre-treated with $CaCl_2$ (5 mM) using cAMP Biotrak EIA system. Aminophylline at 1 nM, a non specific PDE inhibitor, produced less cAMP content (2.3±0.01 fmol/g wet wt) than the control (as shown in Fig. 5.7). Ginger oil (50 µl/100 ml) induced a little increase in cAMP content (5±0.18 fmol/g wet wt), which higher than that of aminophylline, but was not significantly different to the cAMP content when compared with control. 95% of Citral (2.2 mM) reduced cAMP content (2.5±0.08 fmol/g wet wt) which was higher than that of aminophylline and 95% camphene (7.5 mM). This did not reach statistical difference.

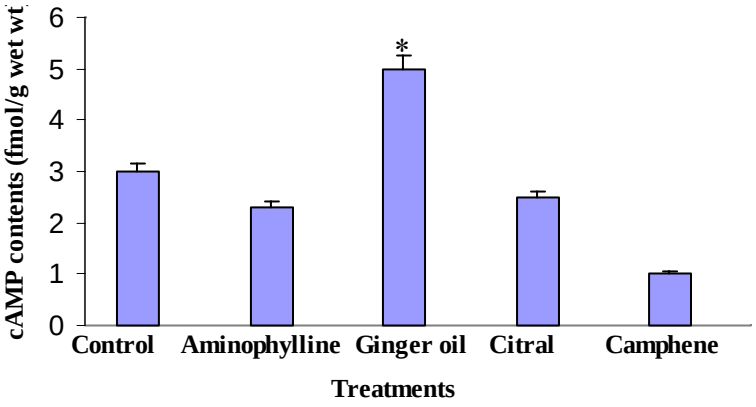

Figure 5.7 cAMP contents of rat myometrium. The strips were incubated in Krebs' solution and 5 mM Ca^{2+}-precontracted for 10 min before (30 min) of treatments; aminophylline (1 nM), ginger oil (50 µl/100 ml), citral (2.2 mM), and camphene (7.5 mM). Vertical bar indicates the S.E.M.*: Significant difference from the respective control with $p < 0.05$.

5.5 Discussion

The aims of this chapter were to examine the inhibitory effects of ginger oil and its pure compounds on COX pathway. To do so, AA was used in the study to generate PGs. Ginger oil and its pure compounds were examined and compared with indomethacin (a specific COX-2 inhibitor). The major measurement of PDE inhibition was measured using a drug, aminophylline (1nM, a PDE inhibitor). The cAMP content was examined after the strips were treated with Ca^{2+} (5 mM $CaCl_2$).

5.5.1 Arachidonic Acid (AA)

The data demonstrated that AA increased myometrial contraction of non-pregnant rat uteri. Ginger oil (50 µl/100 ml), 95% citral (2.2 mM), and 95% camphene (7.5 mM) were able to reduce myometrial contraction induced by AA (20 µM). This suggests that ginger oil and its pure compounds may act on a messenger promoting protein phosphorylation through inhibition of myosin phosphatase enzyme. It is also possible that its metabolites may involve in Rho-Rho-associated Kinase pathway.

5.5.2 COX-2 Pathway

The RIA method was used for measuring PGE_2 concentration occurred via COX-2 pathway in the presence of ginger oil (10^{-5} – 10^{-3} g/ml), 95% citral (10^{-5} g/ml), and 95% camphene (10^{-5} g/ml). The result clearly demonstrated that the hydro-/-steam distillation of ginger rhizomes containing particular citral and camphene, the monoterpenoids: using DMSO solvent, did not inhibit COX-2 enzyme (activity < 50%) of PGE_2 synthesis.

As described previously ginger hydro-distillated containing either predominant citral (neral and geranial), curcumene, α-zingiberene, α-farnesne and β-sesquiphellandrene was identified by GC-MS. The hydro-distillated oil, 95% citral, and 95% camphene: the high content of ginger oil extracted by steam distillation, were not capable to inhibiting effect on COX-2 expression. According to the major constituents of ginger extracts of low concentration of shogaol, had no effect on COX-2 pathway of PG: synthesis (Lantz et al., 2006). Whereas, the extracts from ginger rhizomes containing gingerols or shogaols (identified by HPLC, IC_{50} < 0.1 μg/ml) were highly active at inhibiting LPS-induced PGE_2 production (Lantz et al., 2006). Similarly to the effects of ginger extracts with dichloro-methanol (1:1 v/v) was able to inhibit LPS induced production of PGE_2 and compared with indomethacin at IC_{50} (< 0.1 μg/ml) that suggested by Kuo et al. (2004). Additionally, the ginger extracts have been reported as an anti-inflammatory that it has been suggested by Altman and Marcussen (2001) for effecting at reduction of the ginger extracts on symptoms of arthritis in humans.

5.5.3 Indomethacin

The non-steroidal anti-inflammatory drug (NSAID), indomethacin (20μM) inhibited rat myometrial contraction *in vitro.* Combination of indomethacin with ginger oil and its pure compounds on the following were assessed in rat myometrium. Combined drugs are significantly decreased the contractile response. Similarly, the great responsibility of indomethacin (10^{-5} - 10^{-3} mol/l) administration inhibits the uterine activity by slow release system (Garza et al., 2004). In addition, indomethacin was previously reported that it reduced PGE_2 production by 82% and 90% at the same

concentration (Sawdy et al., 1997). Recently, Sawdy et al. (1998) reported that both drugs, nimesulide (100 μM) and indomethacin (300 μM), inhibited myometrial contractility via mechanisms independent of COX inhibition and also blockaded the Ca^{2+} current. Additionally, these data agree with several previous reports which showed that indomethacin although more potent against COX-1, also inhibits COX-2 with micromolar (Mitchell et al., 1993).

In conclusion, the results suggested that the inhibitory effect of indomethacin combined with ginger oil and its pure compounds on rat myometrial contractile activity could be due to a dual mechanisms of action; 1) inhibiting uterine contractility through the Ca^{2+} channel blocking mechanism and/or 2) prostaglandin synthesis through inhibition of COX-2 pathway with indomethacin.

5.5.4 PDE Pathway

cAMP plays an important for secondary messenger in regulating muscle contraction. In smooth muscles, an increase in cAMP stimulated by a β_2-adrenoceptor agonists causes relaxation. There are three machanisms of relaxation; 1) activation ATPase pump on SR membrane, 2) inhibition of Ca^{2+} slow channels, and 3) inhibition of MLCK (Adelstein et al., 1978; 1982). These are similar to the nature of ralaxing effect attributed to several mechanisms leading to a decrease in both the cytoplasmic free Ca^{2+} concentration and the Ca^{2+} sensitivity of the contractile activity (Savineau and Mironneau, 1990). The inhibitory effects of antagonists on adenosine receptor of PDE enzyme in smooth muscle depressed lower cellular cAMP levels (Laifer et al., 1986; Wolffada et al., 1981).

Several investigations have reported that cAMP inhibited Ca^{2+}-induced contraction of smooth muscles. This is due to the activation of cAMP-dependent protein kinase, producing phosphorylation of the MLCK. It results in an inhibition of actin-myosin interaction (Kerrick and Hoar, 1981; Ruegg et al., 1981; Ruegg and Paul, 1982). However, the inhinitory effect of cAMP was demonstrated under special conditions using high concentrations of cAMP (> 10^{-4} M) by the use of protein kinase. In contrast, the cAMP (3 μM) had no effect on the Ca^{2+}-induced contraction in the smooth muscle and in the presence of exogenous protein kinase. Since this problem is very important for understanding the Ca^{2+} regulation in smooth muscle, further investigations are requirement for studies.

In summary, the major aim of this Chapter was to examine whether the inhibitory effects of ginger oil and its pure compounds on PDE enzyme (the regulator of cAMP production). To detect cAMP content in the treatment of aminophylline, ginger oil, citral, and camphene expressed to 2.3, 5, 2.5, and 1 (fmol/g wet wt, respectively) compared with the control (3 fmol/g wet wt). These results showed inhibitory effects of ginger oil and its pure compounds on rat myometrial contraction may be not mediated by cAMP.

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CHAPTER VI

EFFECTS OF GINGER OIL, CITRAL, AND CAMPHENE ON RAT MYOMETRIAL HISTOLOGICAL CHANGES

6.1 Abstract

As shown in previous Chapters, ginger oil and its pure compounds altered uterine contraction, irrespective how it was produced. To assure that the inhibitory effects of ginger oil and its pure compounds did not occur by damaging of myometrial tissue, the aim of this Chapter was therefore to investigate the effect of ginger oil and its pure compounds on uterine histology.

To do so non-pregnant Wistar rats (200 – 250 g) were humanely killed by cervical dislocation. Myometrial tissues were removed from the rat uteri and immediately immersed in buffered physiological Krebs' solution as a control and others in Krebs' solution containing ginger oil (50 µl/100 ml), 95% citral or 95% camphene, respectively. The tissues were incubated for 30 min at room temperature. These strips were then immediately fixed in 10% formaldehyde and stained to routine histological techniques for light microscopic (LM) and transmission electron microscopic (TEM) studies. The results showed that ginger oil and its pure compounds did not affect myometrial histology in terms of smooth muscle cells, density of intracellular organelles, and subcellular distribution of myofilaments. However, the two pure compounds seemed to have little effect on uterine histology. It was found that few cells of myometrial tissues had slight changes in the density of

extracellular myofilaments, but they exhibited considerable enveloping cellular organelles as sarcoplamic reticulums (SRs) or intracellular myofilaments. Thus, ginger oil and its pure compounds may not affect on myometrial morphological changes as detected with LM and TEM.

6.2 Introduction

As described in Chapter I, most of the uterine wall is composed of myometrial smooth muscle cells, known as myometrial layer. The presence or absence of myometrial lesion may reveal morphological signs of smooth muscle cell changes and can be observed using LM and TEM. The ultrastructural characteristics of the smooth cells, especially the subcellular distribution of, and relationship between, the myofilaments and cellular organelles are of prime interest. The spatial distribution of the SR, the main intracellular store thought to provide Ca^{2+} for myofilament activation in smooth muscle cells, is particular importance relation to contractile properties of the cell (Sweeney et al., 2005; Taggart and Morgan, 2007). The muscles are composed of many subunits known as myofibrils, which can be observed under the electron microscopy (EM). These myofibrils are approximately 1 micrometer (1μm) in diameter and extend in parallel rows from one end of the other organells, such as mitochondria and intracellular membranes, are restricted to the narrow cytoplasmic spaces that remain between adjacent myofibrils. Each myofibril contains even smaller structures called myofilaments, or simply filaments. The thick filaments are primarily composed of the protein myosin, and the thin filaments are primarily composed of the protein actin.

Ginger is pharmacologically safe regarding the investigated aspects (Weidner and Sigwart, 2000). Chinese ginger extract and major pungent principles have been shown to exhibit a verity of biological activities. They found that five new diarylheptanoids and gingerol-related compounds possess significant cytotoxicity against human promyelocytic leukemia (HL-60) cells. An *in vitro* study on the cytotoxic and apoptotic activities of these compounds is associated with the cell apoptosis (Wei et al., 2005). They suggested that the inhibitions of ginger-related compounds had also possessed of activator protein 1 (Lee and Surh, 1998; Surh and Lee, 1998; Bode et al., 2001). Furthermore, the pungent, [6]-paradol and its synthetics [10]-paradol, [3]-dehydroparadol, [6]-dehydroparadol and [10]-dehydroparadol, could induce apoptosis of an oral squamous carcinoma KB cells (Keum et al., 2002). In addition, super-critical CO_2 extracts of ginger against human cancer cell lines (Leal et al., 2003) and antioxidant activity (Tjendraputra et al., 2001). Indeed, most ginger constituents with biological activities posses such a functionality (Surh et al., 1998; Keum et al., 2002; Kim and Kim, 2004).

As mentioned in Chapter I, there are two main organelles related with myometrial contractility, the myofilament and the SR. Myosin, the thick filament consists of MLC that it involve in the regulatory of Ca^{2+}-Ca MLCK phosphorylation and response to diverse contractile stimuli. The thin filament, actin can activate myosin ATPase activity in Ca^{2+}-dependent manner indicating an alternative pathway to MLC_{20} phosphorylation. SR is abundant in uterine smooth muscle cells. Although the major source of activator Ca^{2+} in the myometrium is extracellular fluid, SR is a minor one of its role in excitation-contraction coupling in smooth muscle (Peachey and Porter, 1959; Shmygol et al., 1998; Wray et al., 2001). In order to assess the

morphological changed in the biopsy of the same piece using TEM and LM were used in subsequent procedure of the study (Romanini, 1994). Ultrastucturally, the sarcoplasm of the myometrium is dominated by dense longitudinally aligned contractile filaments containing the contractile proteins, actin and myosin (Romanini, 1994). Structural integrity of the myometium is thus fundamental to normal uterine contractions. Normal myometria, fibromyomata and host myometria are all similar in that do not undergo ultrastructural change during the normal menstrual cycle. Estrogen and progesterone may induce some structural change in the myometrium, during the pre-and post-ovulated phases respectively (Friederici and Decloux, 1968). In contrast, the structural abnormality of the plasmalemmal dense bands that occurs in the presence of fibromyomata may cause to cell disruption. This affected in the normal calcium cycling of the effected myocytes resulting in a disruption of the rhythmical contraction process (Richard et al., 1998). Further suggests that the normal contractile mechanisms and patterns may be disrupted in the presence of myometial irritability (Richard et al., 1998).

However, the effect of ginger oil and its pure compounds on myometrial histological changes has not been elucidated. It is unclear whether the inhibitory effects of ginger oil and pure compounds as shown in previous Chapters occurred by tissue damaging or by physiological mechanisms. The aim of this study was to examine the effects of these treatments on rat myometrial smooth muscle cell histological changes using LM and TEM techniques.

6.3 Materials and Methods

Whole uterus from Wistar rats (as described in Chapter II) were washed with normal saline and incubated in Krebs' solution (control group), Krebs' containing ginger oil (50 µl/100 ml), citral (2.2 mM) or camphene (7.5 mM) at room temperature for 30 min and then fixed in 10% formalin until analyzed. This part of work was conducted at Department of Biology, Faculty of Science, Mahasarakham University, Thailand.

6.3.1 LM Staining Preparations

The horns of rat uteri were cut into short segmenta. These strips were dehydrated in an ascending ethanol series (75%, 85%, 95%, 100% and 100%, 1 hr each) and then removed the sections into pure xylene for 2 min. The sections were embedded with xylene : paraplast (3 : 1 and 1 : 1, 15 min each) and then followed by the pure paraplast for 1 hr. Post-embedded tissues were cut approximately 5 µm with microtome and moved these sections into water bath for incubation (60°C). The tissues were mounted on slides in the slide warmer at 60°C and then twice immersed the slides in pure xylene (5 and 2 min, respectively). Next, tissues were hydrated in a descending ethanol series (100%, 95%, 70%, 50%, 30%, distill water, 2 min each) and stained with heamatoxylin according to standard protocols. All tissues were dehydrated in a descending ethanol series (35%, 50%, 70%, 95%, 2 min each) and stained with eosin for 1 min. Finally, the slides were immersed into 95% ethanol (2-3 min) and 100% ethanol (1 min), pure xylene (5 min) and then covered with cover slip after xylene clearing for LM study.

6.3.2 TEM Staining Preparations

The horns of rat uteri were cut into short segmenta. These strips were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 overnight at 4°C then washed in the same buffer 3 times. Post-fixed with 1% osmium tetroxide for 2 hr then rinsed in distill water 3 times, Gold coated uterus on stubs were examined for TEM, after post-fixing the samples were dehydrated in graded acetone at concentrations of 20%, 40%, 60%, 80%, 100% and 100%, then infiltrated and embedded in Spur's resin. Upon identifying suitable areas from the semithin sections, ultrathin sections (<100 nm thick) of mainly transverse and longitudinal orientation to the uterine tissues were cut with diamond knife, mounted on copper grids then stained with lead citrate and uranyl acetate. The ultrathin sections on copper grids were examined with TEM (JEM 1230) at Central Instrumentation Unit, Faculty of Science, Mahasarakham University (Phungnoi and Narkkong, 2007).

6.4 Results

6.4.1 LM Observation and Uterine Morphological Feature

The rat uteri were stained (with heamatoxylin, eosin) and examined by LM (Fig. 6.1), the only myometrial layer is presented at a higher magnification in Fig. 6.2 – 6.4. It can be seen that the uterus is a thick-walled organ, whose wall consists of three layers; 1) The external serosa (S) is unremarkable and is not damaged by the effects of ginger oil and its pure compounds (Fig. 6.1), 2) The thick myometrium (M) is composed of smooth muscle, subdivided into three poorly delineated layers (outer longitudinal, middle circular and inner longitudinal) and will be described in 6.4.1.1,

3). The endometrium (E) is subdivided into a basal layer and a functional layer as shown in 6.4.1.2.

6.4.1.1 The Thick Myometrium

The basic functional unit of the myometrium is the smooth muscle cell, but these in turn are arranged into bundles of connective tissue interspersed with microvasculature (Wetzstein and Renn, 1970; Word et al., 1993). The thin outer and inner layers are mostly longitudinal or oblique fibers. As seen in Figure 6.1B the tissue treated with ginger oil (50 µl/100 ml) is depicted to dominate by dense longitudinally aligned myofilaments as normal tissue (Fig. 6.1A). Observation of section-treated with 95% citral (2.2 mM, Fig. 6.1 C) contained mildly myofilaments and loose connective tissue under light microscope. Structural integrity of the myometrium-treated with 95% camphene (7.5 mM) is slightly appeared longitudinal myofilaments (Fig. 6.1D).

As described above in Figure 6.1B, the tissue treated with ginger oil (50 µl/100 ml) is densely packed with cells and its illustrated similarly to normal untreated tissue (Fig. 6.1A). The epithelial lining is clearly evident, composed of different cell features, a thin peg cell, which bears no cilia. The photograph (Fig. 6.1C) is lower to show the cellular epithelially lined loose connective tissue, but higher dense cellular epithelium is shown in Fig. 6.2D.

6.4.1.2 The Endometrium

The endometrium is an inner layer defined with the light micoscopy. The endometrial surface consists of non-ciliated epithelium. The sections having taken

during diestrous stage are separated into a deeper basal and a more superficial functional layer, each with its own blood supply. The basal layer, which remains intact during estrous phase, is served by short straight arteries and is occupied by the base of uterine glands. The functional layer served by the arteries, undergoes hormonally modulated cycle changes (Gartner and Hiatt, 2000). The breakdown of the functionalism produces the clinical presentation of the menstrual cycle. Which is a precipitated by luteolysis of the corpus luteum, with drawal of hormonal support, and changes in the vascular supply of the endometrium (Kerr, 1999).

Figure 6.1B, the tissue treated with ginger oil (50 µl/100 ml) is a glandular epithelia layer and located by the simple columnar epithelial cells. Fig. 6.1C, the tissue treated with 95% citral (2.2 mM) is a single fibrotic gland with one layer under columnar epithelial cells. Figure 6.1D, the tissue treated with 95% camphene (7.5 mM) is luminal and glandular epithelial tissues to compare with control (untreated, Fig. 6.1A).

6.4.2 Ultrastructeral Observation and Myometrial Morphological Feature

The ultrathin sections were successfully obtained, involving the structures that the myometrail cells are illustrated by TEM analysis (Fig. 6.2 – 6.4). Ultrastructurally, smooth muscle cells are surrounded by an external lamina (EL) and have placed centrally or perinuclear (N) containing myofilaments. As seen in Figure 6.2A, the normal cell in the Krebs' solution (untreated) is shown by an atypical accumulation of intermediate filaments and perinuclear strips. According to the ultrathin section (in Fig. 6.2B) treated with ginger oil (50 µl/100 ml) contained by the centrally placed nuclei. In contrast to the normal cell, the ultrathin section treated with 95% citral (2.2

mM, Fig. 6.2C) contained elongated nuclei, but it is not expressed the myofilaments surrounded nuclear membrane. It also located in the external lamina. In this case of Figure 6.2D, the ultrathin section treated with 95% camphene (7.5 mM) contained the centrally elongated nuclei and intracellular organelles.

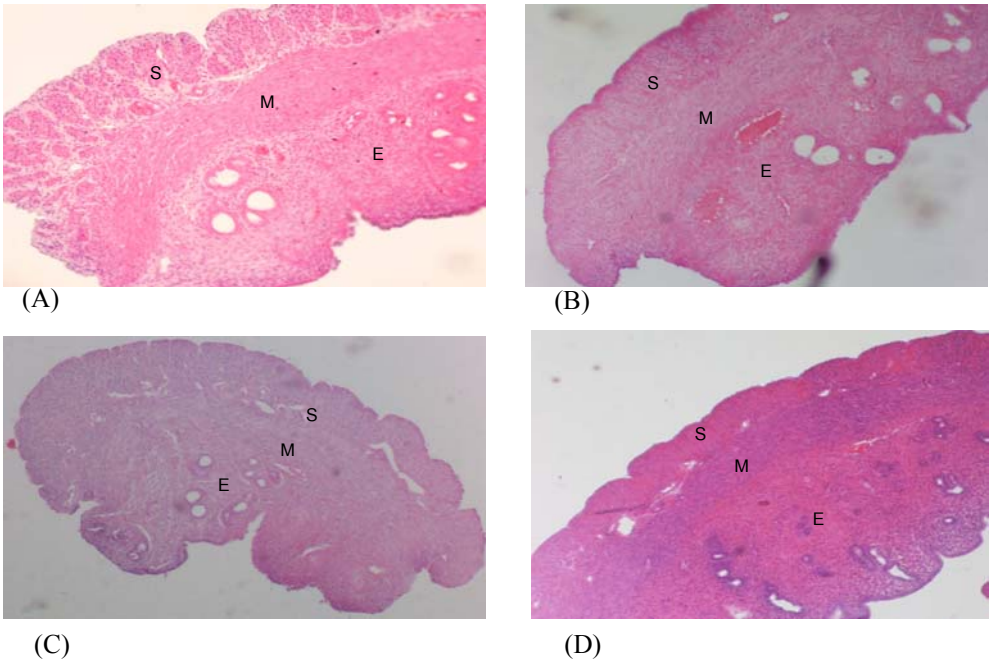

Figure 6.1 The light micrographs image of cross-section tissues of the rat uterus. The control tissue immersed in Krebs' solution pH 7.4 (untreated), it consists of three layers: serosa (S), myometrium (M) and endometrium (E). (A) Normal tissue in the Krebs' solution (untrated) is shown. (B) The tissue trated with ginger oil (50 µl/100 ml) is densely packed by myofilaments. (C) The tissue treated with 95% citral (2.2 mM) contains mildly myofilaments. (D) The tissue treated with 95% camphene (7.5 mM) is slightly appeared longitudinal myofilaments.

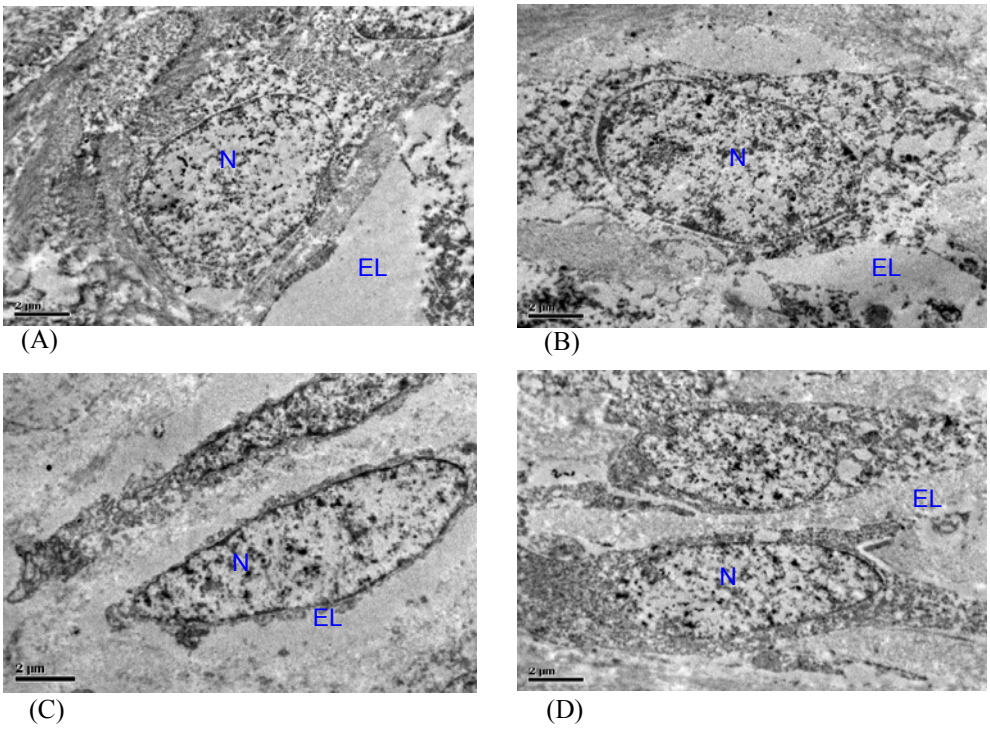

Figure 6.2 Electron micrographs of the general features on extracellular myometrial smooth muscle cells are surrounded by an external lamina (EL). (A) Normal cell in the Krebs' solution (untreated) is shown. (B) The tissue treated with ginger oil (50 µl/100 ml) contained the centrally placed nucleus. (C) The tissue treated with 95% citral (2.2 mM) contains elongated nuclei in the external lamina. The tissue (D) treated with 95% camphene (7.5 mM) contains elongated nuclei and placed in an external lamina.

Ultrastructures of the intracellular myometrial smooth muscle cells show numerous intracellular organelles (Fig. 6.3). No difference of ultrathin sections of the treatments and normal cell (untreated) were observed by TEM magnifications. As seen in Figure 6.3B, the ultrathin section treated with ginger oil (50 µl/100 ml) contained the centrally placed nucleus or myofilaments. As shown in Figure 6.3C, the ultrathin section treated with 95% citral (2.2 mM) contained elongated nucleus, mitochondria (M) and vesicle (V). Figure 6.3D shows the ultrathin section treated with 95% camphene (7.5 mM) contained as vesicle (V), sarcoplasmic reticulum (SR) surrounded nuclear membrane (NM). Most of the label is presented within the sarcoplasm, while relatively small amounts were also observed within the nucleus.

Electron micrographs, a similar distribution of the intracellular organelles in cytoplasm of myometrial smooth muscle cells (Fig. 6.4B, D), were shown thought the largest amount of SR. While the presence in Figure 6.4C and D shown numerous vesicles lower SR than normal section. These organelles were located closely apposed at the surface of smooth muscle cells.

At all diestrous stages, the rat myometrial tissues were characterized by a layer of longitudinally oriented smooth muscle cells, an intracellular organelle nearly nuclear membrane zone containing SR or vesicle (V). As shown in Figure 6.4B and D treated sections with ginger oil (50 µl/100 ml) and camphene (2.2 mM) were similarly composed with SR. They are compared with the normal of ultrathin-untreated section, while the ultrathin section treated with citral (2.2 mM) is more composed with vesicle in the nuclear membrane zone (Fig. 6.4C).

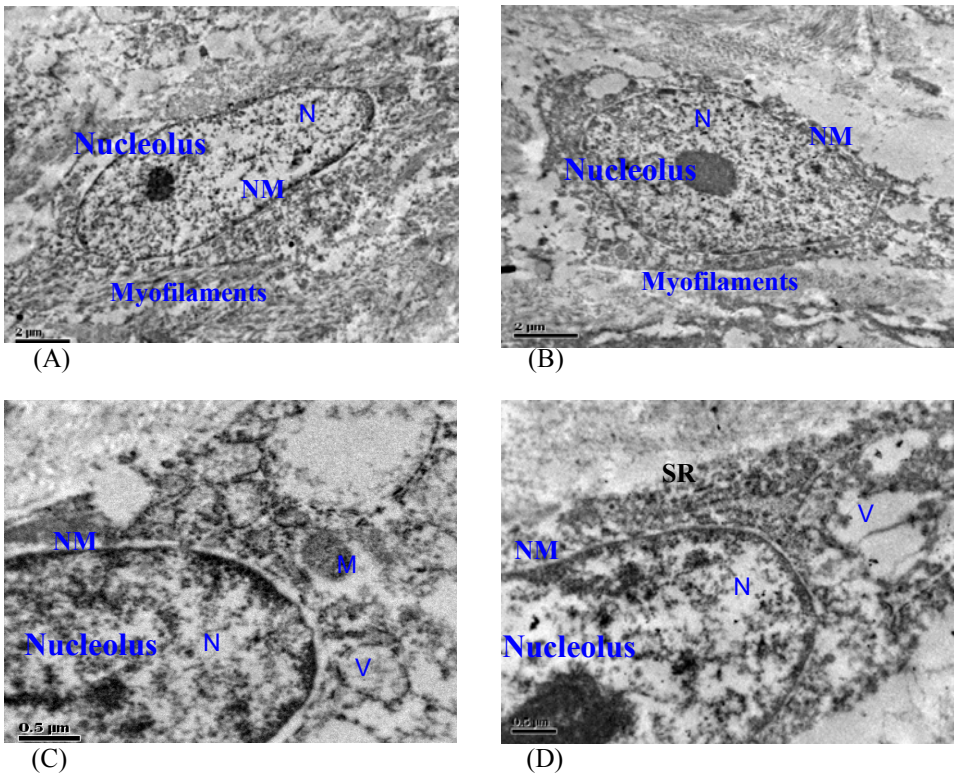

Figure 6.3 Electron micrographs of the intracellular myometrial smooth muscle cells show nucleolus, elongated nuclei (N) and several intracellular organelles. (A) Normal cell in the Krebs' solution (untreated) is shown. (B) The ultrathin section treated with ginger oil (50 µL/100 ml) contained the centrally placed nucleus and myofilaments. (C) The ultrathin section treated with 95% citral (2.2 mM) contains elongated nucleus, mitochondria (M) and vesicle (V). (D) The ultrathin section treated with 95% camphene (7.5 mM) contains as vesicles (V), sarcoplasmic reticulum (SR) surrounded nuclear membrane (NM).

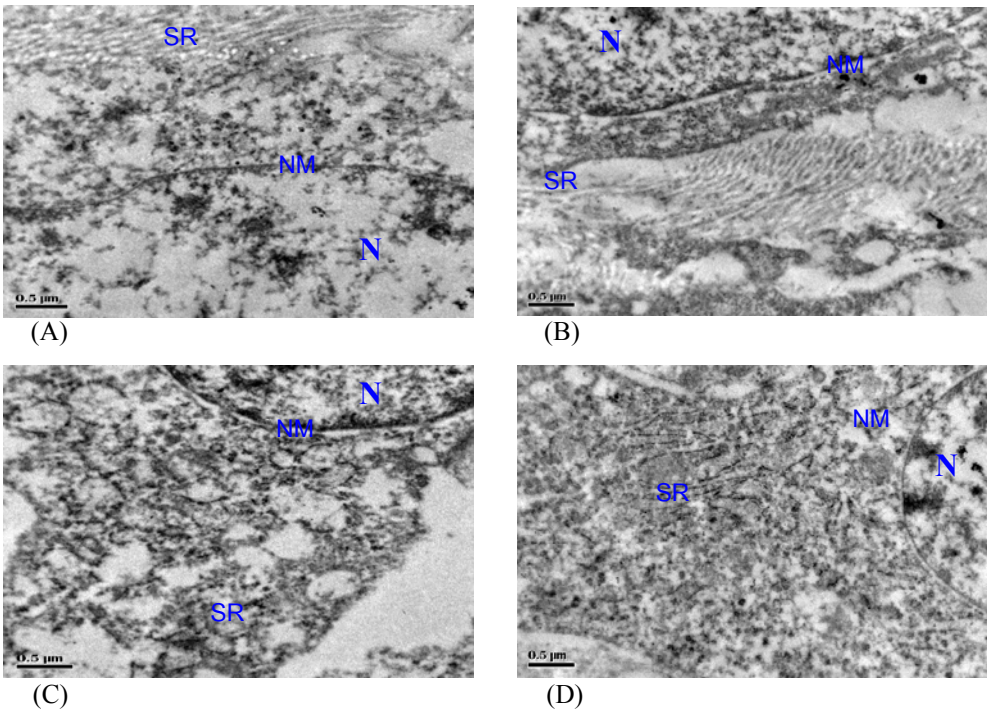

Figure 6.4 Electron micrographs of the intracellular organelles of myometrial smooth muscle cells contain dense organelles. They depict as terminal sacs of sarcoplasmic reticulum (SR) or vesicle (V) lies closely apposed to nuclear membrane (NM) at the surface of myometial smooth muscle cells. (A) normal cell in the Krebs' solution (untreated), (B) the ultrathin section treated with ginger oil (50 µl/100 ml), (C) the ultrathin section treated with 95% citral (2.2 mM) and (D) the ultrathin section treated with 95% camphene (7.5 mM).

6.5 Discussion

This Chapter provided the study of the myometrial histological changes in non-pregnant rat uterus in the presence and absence of ginger oil and its pure compounds. The structural studies with the LM indicated that there was no change on the features of rat uteri during exposure to ginger oil and its pure compounds for 30 min. Ultrastructurally, the results showed histological features, well described by TEM. The features of intracellular myometrial smooth muscle cells, in particular, the SR apposes to have nuclear membrane zone. This finding is consistent with ultrastuctural of smooth muscle cells studied by Steven and Lowe (1997). It showed loosely contractile proteins as myofilaments placed in the sarcoplasm (as seen in Fig. 6.4). These results clearly support those experiments in previous Chapter indicating that the inhibitory effects of ginger oil and its compounds on rat myometrium were recovered to exert potentially contractile activity after washing with BAS (20 μM) because of a great cell responsibilities and healthy.

They have been reported that smooth muscle cells were mediated by a dispersed arrangement of myofilaments (actin and myosin) and terminal sacs of Ca^{2+} ion-containing SR terminate beneath the cell membrane close to its vesicles (Steven and Lowe, 1999). As described in Chapter I, the role of both organelles, SRs and myofilaments, associated with intracellular Ca^{2+} signaling to stimulate MLCK contributing contractile activities of the regulatory light chain on myosin (thick filament) of myometrail smooth muscle cells. Although the smooth muscle SR appeared continuous, its Ca^{2+} stores were organized into spatially distinct units that could have specific physiology (Shmygol and Wray, 2004). However, in the recent study has been reported that the SR acted to limit contractions and acted as Ca^{2+} sink,

rather than to amplify contractions (Kupittayanant et al., 2002). In addition, the thin filament regulation of contraction has been reported. The contraction of myometrial smooth muscle cells is mediated by a dispersed arrangement of actin and myosin (Steven and Lowe, 1997) in cytoplasm and dominated by dense longitudinally aligned contractile filaments (Romini, 1994; Berne et al., 1998). As previously mentioned, smooth thin filament contained two specific proteins caldesmon and calponin. Both proteins inhibit ATPase activity of acto-myosin and the effect is being reversed by phosphorylation of these proteins (Allen and Walash, 1994).

There have previously reports showing that ginger was listed in modern pharmacological safe (Weidner and Sigwart, 2000) about its anti-inflammatory effects, antioxidation (Srivatava and Mustafa, 1989; Peng, 1992; Aesehbach et al., 1994; Habash et al., 2000; Ahmed et al., 1999; 2000; Surh, 2003). Additionally, extract of ginger (50 mg/kg body weight) has been shown to antioxidant and anti-carcinogenic (Manju and Nalini, 2005). In a similar study, ginger had better therapeutic than prophylactic detoxication effects on liver cadmium accumulation (Egwurugwu et al., 2007). There have been no reports of significant side effects or severe toxic reaction following the consumption or exposure of ginger in usual therapeutic doses. This fact the use of ginger for thousands of years by many different cultures confirm to its safely (Weidner and Sigwart, 2000). Ginger contains a number of different pungent and active ingredients, steam distillation of fresh ginger produced ginger oil which contains a high portion of momo-and sesqui-terpene hydrocarbons (as show in Table 2.1) predominantly citral and camphene. The study of citral has been presented that it was tested to the best catalysis of menthols or menthol mixture (Trasarti et al., 2004). Because of its strong lemon flavor and odor, citral is used as a

flavoring and fragrance agent in foods and cosmetics. It is a generally recognized as safe (GRAS) list chemical. The average daily intake of citral in human was estimated to be 5 mg/kg (Council of Europe, 1974). It is present in chewing gum, baked goods, candy, ice-cream, and in beverages at concentrations ranging up to 170 ppm (Opdyke, 1979). Several studies supported this study in non-pregnant rat uterus with citral experimentations. Additionally, the study on the embryofeto-toxicity of citral in rat increased ratio of resorptions per implantations at 60 and 125 mg/kg body p.o., and impaired implantation in doses higher than 125 mg/kg p.o. as well (Ana Cristina et al., 1995). Having found another constituent of ginger oil, camphene did not affect on uterine morphology as the histological features are very similar to those other treatments. Camphene is one of chemical, which is attractive by its uses on pharmaceutical and cosmetic applications.

As discussed above, the result of myometrial smooth muscle cells with immersed into ginger oil (50 µl/100 ml) and its compounds (95% citral 2.2 mM and 95% camphene 7.5 mM) may also remain to raise a great responsibility function as similar to the normal (untreated) tissue. These treatments may not affect on myometrial morphological changes using LM and TEM observation. Although, the effects of two pure compounds are mildly lower density of extracellular myofilaments in only few cells than untreated cells, they are also to remain response of their functions and containing with organelles.

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CHAPTER VII

CONCLUSION

The main aims of this thesis were to investigate the effects of ginger oil and its pure compounds on uterine contraction and determine the mechanisms where by ginger oil and its pure compounds exert their effects. The major findings are summarized as follows:

7.1 Identification of Ginger Oil

Ginger oil was analyzed by GC/MS. It contains mainly eight compounds with retention times of 5.70 (7.74%), 7.43 (7.44%), 12.35 (10.00%), 13.03 (14.49%), 17.61 (4.71%), 17.88 (16.22%), 18.09 (6.80%), 18.09 (6.80%) and 18.45 (5.70%). They are camphene, *β*-phellandrene, neral, geranial, curcumene, α-zingiberene α-farnesene and *β*-sesquiphelladrene, respectively. Traces of twenty-three other known compounds and two unknown compounds were found.

7.2 Effects of Ginger Oil and Its Pure Compounds on Spontaneous Contraction and PGs

Under control conditions, the spontaneous contraction of rat myometrial smooth muscle can be inhibited by the effects of the different concentrations of ginger oil and some of its pure compounds (citral 2.2 mM and camphene 7.5 mM). Ginger oil in a concentration dependent manner (10 – 150 μl/100 ml) inhibited myometrial

contraction arising either by spontaneously or PGs-induced contraction. It showed similar effects to citral on spontaneous and PGs-induced contraction. Camphene, one of ginger oil constituents, can weakly inhibit uterine contraction. IC_{50} of ginger oil, citral, and camphene was found at 50 µl/100 ml, 2.2 mM, and 7.5 mM, respectively. In the absence of external Ca^{2+}, PGs ($PGF_{2\alpha}$/PGE_2) elicited a small force. This force was inhibited by ginger oil, citral and camphene. Thus the inhibitory effects of ginger oil and its pure compounds on uterine contraction may be via inhibition of Ca^{2+}-CaM MLCK pathway. In addition in the absence of external Ca^{2+}, they can inhibit force, presumably via inhibition of non-Ca^{2+}-CaM MLCK pathway.

7.3 Effects of Ginger Oil and Its Pure Compounds on AA

Arachidonic acid (AA) increased, at constant Ca^{2+}, the levels of force and 20 KDa myosin light chain (MLC_{20}) phosphorylation in permeabilized (Gong et al., 1992). Moreover, AA itself can activate protein kinase C (Sward et al., 2000), having Ca^{2+}-sensitizing effects on smooth muscle.

AA (20 µM) induced myometrial contraction. In the presence of ginger oil (50 µl/100 ml) and 95% citral (2.2 mM) and 95% camphene (7.5 mM), the contractions were decreased. This inhibition of force may be via inhibition of PKC or ROK pathway.

7.4 Effects of Ginger Oil and Its pure compounds on the Mechanism of COX-2 Pathway

PGE_2 biosynthesis is regulated by successive metabolic steps involving the phospholipase A_2-mediated release of arachidonic acid (AA) and its conversion to

PGE_2 by cyclooxygenase (COX) activities. The conversion of AA to PGs in biosynthesis step is mediated by COX-1 and COX-2 (Smith and Marnett, 1991). A common precursor AA, the increase in $PGE_2/PGF_{2\alpha}$ ratio indicates that its synthesis directed preferentially towards the PGF compounds (Downie et al., 1974).

The result show that PGE_2 inhibitor, aspirin, inhibited both COX-1 and COX-2 enzyme, but this was not the case for the ginger oil and its pure compounds. The COX-2 inhibitor, indomethacin, is a non-steroidal anti-inflammatory drug (NSAID) used to reduce PG productions (Sawdy et al., 1998; DeWitt et al., 1991). It inhibited myometrial contractility by blocking Ca^{2+} current. It was found that ginger oil and its pure compounds further inhibited contraction in the presence of indomethacin. This supports that the inhibitory effects of ginger oil, citral and camphene on rat myometrial contractile may contribute to indomethacin mediated COX-2 pathways as well as the blockade to the Ca^{2+} L-type channels as shown in previous Chapters.

7.5 Effects of Ginger Oil and Its Pure Compounds on the Mechanism of PDE Pathway

In most cell types, rapid changes in $[Ca^{2+}]_i$ directly control cellular functions, while slower fluctuations in cAMP levels modulate the Ca^{2+} control system. cAMP is synthesized by adenylyl cyclase and PDE. PDE4 is specific for the hydrolysis of cAMP (Beavo, 1995). It is well known that aminophylline is non-specific PDE enzyme and has been reported to inhibit pregnant and non-pregnant rat uterine smooth muscle contraction (Apadin et al., 1998). The nature of this relaxing effect is attributed to several mechanisms leading to a decrease in both the cytoplasmic free

Ca^{2+} concentration and the Ca^{2+} sensitivity of the contractile machinery (Savineau and Mironneau, 1990).

In uterine smooth muscle, ginger oil, citral, and camphene caused an accumulation of cAMP in low level (5±0.18, 2.5±0.08, 1±0.33 fmol/g wet wt; respectively). This, however, was, higher than that of aminophylline, the inhibitor of PDE (2.3±0.01 fmol/g wet wt). It has been suggested that the concentration of cAMP should be ~10^{-6} M to confirm the effects of PDE pathway.

Thus, the inhibitory effects of ginger oil and its pure compounds on myometrial contraction (with 5mM $CaCl_2$-pretreated) may not be mediated by PDE pathway as they produced lower quantity of cAMP level than 10^{-6} M.

7.6 Effects of Ginger Oil and Its Pure Compounds on Myometrial Histological Changes

Ginger oil and its pure compounds do not affect uterine morphological changes, after incubated the strips with the oil or its pure compounds. This is clearly shown by routine histological procedure and observed qualitatively under light microscope (LM) and transmission electron microscope (TEM). Observation of microscopic feature of the myometrail smooth muscle cells shows that the cytoplasm of myometrial smooth muscle cells is dominated by dense longitudinally aligned contractile filaments containing the contractile proteins, actin and myosin. Structural integrity of the sections is thus fundamental to normal uterus. Thus it is similar to the control section. The ultra-structure, as observed by TEM in tissues that exposing to ginger oil and camphene, are normal. However, there is a sign of change in extracellular myofilament, SR, and intracellular myofilaments with citral.

Thus, the results in this study, indicate that expression of SR and myofilaments in myometrial smooth muscle cells generally function. Ginger oil and its pure compounds do not damage myometrial tissues as they inhibit contraction. This was also confirmed by the effect of BSA as described previously.

Future Work

In future studies, it would be interesting to further investigate some possible future experiments, for example, to investigate the role of ginger oil on prevention of morning sickness in pregnant women. Morning sickness is one of the most common complaints during pregnancy, a combination of nausea, headache and dizziness that is experienced by about half of pregnant women during their first few months of pregnancy. To relieve this problem, can also make a tasty morning sickness treatment by combining ginger with lemon juice against nausea during the day.

However, the safety of use in pregnant women is not known. This study showed that ginger oil can inhibit uterine contraction in non-pregnant, but not in pregnant rats. Thus, it is worth examining the effects in pregnant rats and women to answer its safety use.

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Table 3.1 The effects of ginger oil at various concentrations on spontaneous contraction.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Ginger oil (µl/100ml)				
0 (control)	100	100	100	
10	89.48±5.64	93.14±3.18	90.89±4.12	5
20	88.86±6.91	91.53±3.76	83.72±7.11	5
30	81.04±6.43*	84.22±5.38*	72.84±7.74*	5
40	67.61±9.03*	83.28±3.88*	70.94±8.29*	5
50	49.03±3.10*	77.55±1.53*	50.69±3.66*	5
70	40.35±3.60*	70.55±4.69*	42.79±5.24*	5
100	31.08±7.55*	64.72±4.47*	37.64±6.64*	5
150	25.19±5.86*	32.00±4.42*	29.64±5.89*	5

The *p*-values for amplitude, frequency, and area under the curve of ginger oil treated are significantly different from the control (* $P < 0.05$). Mean value±S.E.M are given; *n* is number of animals.

Table 3.2 The effects of $PGF_{2\alpha}$-and PGE_2-induced contraction.

	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
$PGF_{2\alpha}$-induced contraction				
control	100	100	100	5
$PGF_{2\alpha}$	128.42±5.13*	128.37±3.19*	156.21±2.96*	5
$PGF_{2\alpha}$ + ginger oil	56.39 ± 5.34*	81.24±3.68*	56.49±4.28*	5
PGE_2-induced contraction				
control	100	100	100	5
PGE_2	128.17±5.48*	154.89 ±2.38*	179.49 ± 2.22*	5
PGE_2 + ginger oil	50.65±6.99*	58.64±0.10*	57.61±2.48*	5

The *p*-values for amplitude, frequency, and area under the curve of ginger oil treated are significantly different from the control (*$P < 0.05$). Mean value±S.E.M are given; *n* is number of animals.

Table 3.3 The effects of ginger oil on $PGF_{2\alpha}$-and PGE_2-induced uterine contraction in the continued presence of high Ca^{2+}.

	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Spontaneous contraction *control*	100	100	100	
$PGF_{2\alpha}$-induced contraction				
$PGF_{2\alpha}$	100	100	100	5
$PGF_{2\alpha}$ + ginger oil	48.71±5.04*	71.75±3.21*	46.33±6.22*	5
$PGF_{2\alpha}$ + ginger oil + 5mM $CaCl_2$	120.77±3.24*	123.59±6.56*	117.99± 4.05*	5
PGE_2-induced contraction				
PGE_2	100	100	100	5
PGE_2 + ginger oil	47.26±5.85*	79.76±3.46*	46.20±5.73*	5
PGE_2 + ginger oil + 5mM $CaCl_2$	103.87±.36*	129.09±11.96*	114.77±5.56*	5

The *p*-values for amplitude, frequency, and area under the curve of ginger oil treated are significantly different from the control (* $P < 0.05$). Mean value±S.E.M are given; *n* is number of animals.

Table 4.1 The effects of citral at various concentrations on spontaneous contraction.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Citral (mM)				
0 (control)	100	100	100	
0.5	87.61±4.40*	86.26±5.73*	75.95 ±4.09*	5
1	75.71±3.26*	83.11 ±3.58*	66.43±4.36*	5
1.6	52.23±3.58*	71.44±4.42*	51.03±4.98*	5
2.2	48.65±4.88*	69.00±5.10*	49.07±3.66*	5
2.7	41.39±2.80*	67.02±1.49*	45.49±3.05*	5
3.8	37.07±2.58*	53.20±3.75*	40.17±3.69*	5
5.6	29.70±3.05*	33.18±5.21*	28.23±4.17*	5
8.3	19.83±2.65*	18.17±2.08*	16.23±5.36*	5

The *p*-values for amplitude, frequency, and area under the curve of citral treated are significantly different from the control (*P<0.05). Mean value±S.E.M are given; *n* is number of animals.

Table 4.2 The effects of camphene at various concentrations on spontaneous contraction.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Camphene (mM)				
0 (control)	100	100	100	
0.5	97.01±3.73	98.41±2.03	98.35±2.59	5
1.0	96.38±3.79	96.66±4.36	97.44±2.61	5
1.5	83.81±4.16*	84.87±1.16*	84.3±2.50*	5
2.0	81.86±5.03*	83.85±0.22*	83.85±0.20*	5
2.5	73.67±2.71*	80.78±1.63*	75.30±2.44*	5
3.5	70.52±4.75*	78.43±0.19*	64.26±4.79*	5
5.0	58.33±1.83*	74.08±1.44*	62.15±1.61*	5
7.5	53.37±3.53*	50.64±2.51*	53.42±3.21*	5

The *p*-values for amplitude, frequency, and area under the curve of camphene treated are significantly different from the control (*P<0.05). Mean value±S.E.M are given; *n* is number of animals.

Table 4.3 The effects of citral and camphene on spontaneous contraction in the continued presence of high Ca^{2+}.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Spontaneous contraction				
control	100	100	100	
citral	41.39±2.80*	67.02±1.49*	45.49±3.05*	5
citral + 5mM $CaCl_2$	108.93±3.74*	141.29±1.06*	135.85±1.88*	5
Spontaneous contraction				
control	100	100	100	
camphene	73.67±2.71*	80.78±1.63*	75.30±2.44*	5
camphene + 5mM $CaCl_2$	113.72±3.65*	128.62±3.42*	135.76±2.68*	5

The *p*-values for amplitude, frequency, and area under the curve of citral / camphene treated are significantly different from the control (*P<0.05). Mean value±S.E.M are given; *n* is number of animals.

Table 4.4 The effects of $PGF_{2\alpha}$-and PGE_2-induced uterine contraction.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
$PGF_{2\alpha}$-induced contraction				
control	100	100	100	5
$PGF_{2\alpha}$	134.78±4.07*	119.34±3.61*	132.88±4.15*	5
$PGF_{2\alpha}$ + citral	59.51±2.50*	62.80±1.06*	66.44±2.25*	5
PGE_2-induced contraction				
control	100	100	100	5
PGE_2	114.27±4.91*	154.98±1.62*	115.98±4.59*	5
PGE_2 + citral	42.87±2.70*	56.50±3.31*	57.44±1.30*	5

The *p*-values for amplitude, frequency, and area under the curve of citral treated are significantly different from the control (*$P<0.05$). Mean value±S.E.M are given; *n* is number of animals.

Table 4.5 The effects of $PGF_{2\alpha}$-and PGE_2-induced uterine contraction.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
$PGF_{2\alpha}$ –induced contraction				
control	100	100	100	5
$PGF_{2\alpha}$	112.85±3.81*	122.34±3.19*	142.10±2.01*	5
$PGF_{2\alpha}$ + camphene	77.32±2.42*	78.21±2.60*	87.29±2.29*	5
PGE_2-induced contraction				
control	100	100	100	5
PGE_2	109.78±1.02*	111.93±4.69*	122.72±3.95*	5
PGE_2 + camphene	78.55±1.32*	73.91±4.62*	84.89±4.93*	5

The *p*-values for amplitude, frequency, and area under the curve of camphene treated are significantly different from the control (*P<0.05). Mean value±S.E.M are given; *n* is number of animals.

Table 4.6 The effects of citral on $PGF_{2\alpha}$-and PGE_2-induced uterine contraction in the continued presence of high Ca^{2+}.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
$PGF_{2\alpha}$-induced contraction				
$PGF_{2\alpha}$	100	100	100	5
$PGF_{2\alpha}$ + citral	57.78±4.12*	60.65±4.43*	55.18±4.58*	5
$PGF_{2\alpha}$ + citral + 5mM $CaCl_2$	122.43±3.38*	104.90±2.02	116.17±3.35*	5
PGE_2-induced contraction				
PGE_2	100	100	100	5
PGE_2 + citral	42.87±4.70*	56.50±3.31*	57.44±1.30*	5
PGE_2 + citral + 5mM $CaCl_2$	106.33±2.44*	107.48±2.27*	124.40±4.90*	5

The *p*-values for amplitude, frequency, and area under the curve of citral treated are significantly different from the control (*P<0.05). Mean value±S.E.M are given; *n* is number of animals.

Table 4.7 The effects of camphene on $PGF_{2\alpha}$-and PGE_2-induced uterine contraction in the continued presence of high Ca^{2+}.

Concentration	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
$PGF_{2\alpha}$-induced contraction				
$PGF_{2\alpha}$	100	100	100	5
$PGF_{2\alpha}$ + camphene	64.64±3.82*	60.51±3.12*	68.53±3.55*	5
$PGF_{2\alpha}$ + camphene + 5mM $CaCl_2$	106.62±1.67*	115.43±2.35*	108.79±0.61*	5
PGE_2-induced contraction				
PGE_2	100	100	100	5
PGE_2 + camphene	74.31±4.28*	68.93±1.90*	70.21±2.96*	5
PGE_2 + camphene + 5mM $CaCl_2$	104.60±1.80*	109.39±3.68*	110.63±3.38*	5

The *p*-values for amplitude, frequency, and area under the curve of camphene treated are significantly different from the control (*P<0.05). Mean value±S.E.M are given; *n* is number of animals.

Table 5.2 The effects of ginger oil, citral, and camphene on AA- induced uterine contraction.

	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Ginger oil (50 µl/100 ml)				
Control (spontaneous contraction)	100	100	100	
AA	116.68±3.17*	136.97±5.38*	128.72±4.73*	5
AA + ginger oil	76.25±7.70*	75.56±4.78*	59.19±8.39*	5
Citral (2.2 mM)				
Control (spontaneous contraction)	100	100	100	5
AA	111.85±2.39*	129.24±4.02*	121.84±4.33*	5
AA + Citral	60.95±4.75*	43.83±2.55*	54.37±1.23*	5
Camphene (7.5 mM))				
Control(spontaneous contraction)	100	100	100	
AA	114.00±2.36*	138.94±4.06*	145.5±8 4.09*	5
AA + camphene	90.46±7.19*	75.17±7.18*	91.63±2.29*	5

The *p*-values for amplitude, frequency, and area under the curve of various treatments treated are significantly different from the control (*$P < 0.05$). Mean value±S.E.M are given; *n* is number of animals.

Table 5.3 The effects of ginger oil, citral, and camphene on uterine contraction in the presence of indomethacin.

	Amplitude (%, Mean±S.E.M)	Frequency (%, Mean±S.E.M)	AUC (%, Mean±S.E.M)	n
Ginger oil (50 µl/100 ml)				
Control (spontaneous contraction)	100	100	100	
indomethacin	68.15±3.58*	60.77±5.56*	66.98±2.00*	5
indomethacin + ginger oil	60.92±3.65*	36.51±8.87*	61.71±7.14*	5
Citral (2.2 mM)				
Control (spontaneous contraction)	100	100	100	
indomethacin	81.85±5.60*	84.84±3.8*	87.86±2.35*	5
indomethacin + citral	58.37±8.55*	63.33±6.12*	50.78±4.00*	5
Camphene (7.5 mM))				
Control(spontaneous contraction)	100	100	100	
indomethacin	84.99±4.60*	71.52±6.19*	70.10±4.35*	5
indomethacin + camphene	68.78±8.47*	50.94±5.16*	64.08±3.39*	5

The *p*-values for amplitude, frequency, and area under the curve of various treatments treated are significantly different from the control (*$P < 0.05$). Mean value±S.E.M are given; *n* is number of animals.