IN VITRO EFFECT OF RUTIN HYDRATE ON ISOLATED PREGNANT MICE UTERINE CONTRACTION

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DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

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CERTIFICATION

This is to certify that this research work titled "*In vitro* effect of Rutin Hydrate on isolated pregnant mice uterine contraction" was carried out by MISS OKONMAH ESTHER CHUKWUNONYELUM, with matriculation number; PHA1606848 in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria, and meets the requirement for the award of the Doctor of Pharmacy (Pharm. D) degree from the aforementioned university.

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DEDICATION

This work is dedicated to God Almighty, whose divine grace, wisdom, and guidance have illuminated every step of this journey. I extend my heartfelt dedication to my parents, whose unwavering support and love have been a continuous source of motivation throughout my academic journey.

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ABBREVIATIONS

RT-Rutin hydrate

OT-Oxytocin

KCL-Potassium chloride

Ca^{2+}-Calcium ions

ATP-Adenosine triphosphate

DAG-Diacyl glycerol

PLC-Phospholipase C

MLCK-Myosin light chain kinase

MLC-Myosin light chain

cAMP-cyclic Adenosine monophosphate

PG-Prostaglandin

MMPs-Matrix metalloproteinases

TNFa-Tissue necrosis factor alpha

IL8-Interleukin-8

PDE-Phosphodiesterase

SR-Sarcoplasmic reticulum

CCBs-Calcium channel blockers

OTR-Oxytocin receptor

PKA-Phosphokinase A

NSAIDS-Nonsteroidal anti-inflammatory drugs

COX-Cyclooxygenase

cGMP-cyclic Guanosine monophosphate

MAPK-Mitogen activated protein kinase

RhoA-ROK/ERK1/2--Rho-associated protein kinase/ “Pro-directed” protein kinases

FP/TP/IP/DP receptors-Prostaglandin F/Thromboxane/Prostacyclin/Prostaglandin D receptors.

EP1/EP2/EP3/EP4 receptors-Prostaglandin E_2 receptor 1/ Prostaglandin E_2 receptor 2/ Prostaglandin E_2 receptor 3/ Prostaglandin E_2 receptor 4.

ABSTRACT

Rutin hydrate (Vitamin P), a known flavonoid glycoside, commercially available and found in various plants, have been shown to possess a range of pharmacological effects including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities. There is however limited scientific knowledge on its effect on the uterus. This study aimed at investigating the effect of rutin hydrate on spontaneous and agonist-induced contractions of isolated pregnant mice uterine tissues.

Pure rutin hydrate sample was examined on uterine tissues isolated from healthy pregnant albino mice (gestation day 18). This investigation was carried out using a range of concentrations (0.03-25mg/ml) to assess its activity on spontaneous contractions, oxytocin-induced contractions, high KCL-induced contractions, as well as oxytocin-induced contractions in a calcium-free medium. For mating conditions to achieve pregnancy, virgin female albino mice were paired with male mouse of the same strain overnight at the ratio of 2:1 and gestation day 0 was defined by the presence of vaginal plug in the paired females the next morning.

Rutin hydrate exerted inhibitory effects on spontaneous uterine contractions in a dose dependent manner. Both the frequency and amplitude of spontaneous contractions progressively reduced until complete elimination. There was immediate tissue recovery following washout of the drug with fresh PSS. It however showed no significant changes to the contractions elicited by oxytocin, high KCL and oxytocin-induced contractions in a zero-calcium medium.

This study offers scientific evidence suggesting that rutin hydrate has relaxing effects on pregnant uterine contractions but does not interfere with calcium-dependent mechanisms of action in the uterus. Hence, this compound merits further investigation as a potentially new or complementary tocolytic drug therapy, via studying its effects on pathways other than calcium antagonism, for managing conditions requiring uterine contractility inhibition during pregnancy, such as in preterm labour.

Keywords: Rutin hydrate, flavonoids, pregnant mice, uterus, *in vitro* study, tocolytics, preterm labour.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

The pregnant uterus undergoes a series of complex contractions throughout gestation, which are crucial for maintaining pregnancy and initiating labour. The contraction of uterine smooth muscles, like those of other smooth muscles, is a complicated process that is governed by involving several multiple signalling pathways and factors, including hormonal, mechanical and neurological factors (Garrett *et al*., 2022). Biological actions carried out by flavonoids in plants, animals, and microbes span a wide range (Panche *et al*., 2016). According to Khodzhaieva *et al*., (2021) although flavonoids are one of the most common families of biological pigments, the precise number of flavonoids that have been found and studied is still unclear. Generally, these compounds have been reported to have a wide range of biological activities, including antioxidant, anti-inflammatory, anticancer, cardioprotective, hepatoprotective, antimicrobial, antiviral, antiallergic, vasodilatory and in the treatment of neurodegenerative diseases (Sangeetha *et al*., 2016). The flavonoid rutin hydrate, present in many plants, has been shown to have a number of pharmacological effects, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective ones (Ganeshpurkar & Saluja, 2017). In 2020, World Health Organization (WHO) reported 287,000 maternal deaths, with 95% in LMICs, particularly in Africa, where many could have been prevented. This chapter therefore serves to evaluate the existing literature on the effect of rutin hydrate, a flavonoid, on the contraction of isolated smooth muscles in order to evaluate its possible potential as a therapeutic agent in obstetrics.

1.1 THE UTERUS: HUMANS AND MICE

1.1.1 HUMAN UTERUS

In humans, the development of the female reproductive system is a complex process. It primarily derives from four origins: mesoderm, primordial germ cells, coelomic epithelium, and mesenchyme. The uterus forms during Mullerian organogenesis accompanied by the development of the upper third of the vagina, the cervix and both fallopian tubes (Moncada-Madrazo & Rodriguez, 2022). According to Kenhub (2023), the paranemesophrenic or Mullerian ducts, which are early foetal components of the female reproductive tract, give rise to the womb (uterus). The uterine endometrium and myometrium grow from the mesenchyme of the genital cord; in fact, the uterine body can be clearly seen as early as nine weeks of gestation. For pregnancy and delivery in mammals, the uterus is a crucial reproductive organ. The uterus is located between the rectum and the urinary bladder in the pelvic cavity of humans. The virgin uterus has a pear shape (pyriform) and is flattened antero-posteriorly (Sembulingam & Sembulingam, 2019). As Kenhub (2023) states, Human uterus is separated into three sections relative to the isthmus. Towards the centre of the uterus, the isthmus lies as a 1 cm long, thin channel. As a result, the uterus is split into the fundus (above the entrance points of the fallopian tube), the corpus or body (below the fundus and on either side of the isthmus) and the cervix (below the isthmus) which protrudes into the vagina. In humans, the uterus is likewise histologically divided into three layers as Sembulingam and Sembulingam (2019) explains: The perimetrium (serous or outer layer): A thin connective tissue layer derived from the peritoneum that anteriorly covers the uterus completely but posteriorly covers only up to the isthmus. The myometrium (middle muscular layer): This is the thickest layer of the uterus made up of smooth muscle fibres arranged in three layers *viz*; The external myometrium which has muscle fibres

arranged transversely, the middle myometrium with muscle fibres arranged longitudinally, obliquely and transversely; and the internal myometrium with muscle fibres arranged circularly. Finally, the endometrium (inner muscular layer): lined with simple columnar epithelium and contains numerous glands. During pregnancy, the uterus undergoes significant changes to accommodate the growing foetus. Elevated progesterone levels maintain a noncontractile state, while oestrogen triggers early uterine growth. Uterine weight increases from 70 g to 1100 g, and volume capacity expands from 10 mL to 5 L. Between weeks 12 and 16, the uterus unfolds, allowing space for foetal growth. Rapid uterine elongation and wall thinning occurs from 20 to 30 weeks, and after delivery, the uterus gradually returns to its pre-pregnancy state (Pascual & Langaker, 2023).

1.1.2 MOUSE UTERUS

Using anatomical and histological techniques, the uterine anatomy of the Swiss albino mice (*Mus musculus*) has been investigated. The uterus type in mice has been found to be duplex, comparable to other small laboratory species like guinea pigs, white albino rats and rabbits. It is distinct from previous known forms that were bipartite or bicornuate as seen in cows and pigs respectively (Rabie & Haibat, 2019). In Avni *et al*., (2012) study on mice, the uterus was stated to be duplex, comprising of two independent uterine horns that unite at the cervix and open into the vagina via two different cervical canals. His research explained that blood enters the mouse uterus caudally by the uterine artery and cranially through the ovarian artery, indicating a dual arterial blood supply inside the mouse uterine horns. This further suggests that depending on where a foetus is located along the uterine horn, it may receive nutrition from one or both routes. The adult female human uterus contains endometrial or uterine glands that are crucial for female

fertility and in the mouse, similar uterine glands are also present in the lateral and anti-mesometrial regions of the uterine horn (Vue & Behringer, 2020).

1.1.3 COMPARISON OF THE HUMAN AND MOUSE UTERUS

Both in the mouse and the human, the female genital system is dynamic and has a variety of morphologic appearances depending on hormonal effects throughout the oestrus/menstrual cycle and pregnancy. While ovaries, fallopian tubes, uteri, and placentation, are present in both humans and mice as mammals, there are several structural and functional variations between both species. Particularly, there are significant differences in the gross anatomy between the two species, whereas the histologic appearance and basic functions appear to be more similar (Rendi *et al*., 2012). For instance, even though the uterus of mice and humans both develop from the bilateral Mullerian ducts in the embryology stage, the degree of fusion of these ducts in the midline differs vastly in both species during their developmental stages. Majority of the Mullerian ducts in humans merge in the midline to produce the uterus, cervix and to aid in vaginal development, with the remaining un-fused sections forming the fallopian tubes. However, in mice, majority of the Mullerian ducts remain unfused to generate the oviducts and uterine horns, with the fused portion creating the cervix and contributing to the vagina (Cunha *et al*., 2019). Although the embryology of the two species is similar, humans and these rodents differ greatly in the fundamental aspects of pregnancy. Typically, humans have a forty (40) week gestation period and deliver one or a few off springs in cases of multiple pregnancies, whereas rodent gestations are shorter (19 to 21 days) with larger litter sizes. The uterus of humans is pyriform (shaped like a light bulb), whereas mice have bicornuate uteri with several implantation sites in each uterine horn. The peculiar three-layer uterine composition is shared by both species (Malik *et al*., 2021).

1.2 UTERINE CYCLES: HUMANS AND MICE

The menstrual cycle in humans and the oestrus cycle in mice are the two main reproductive cycles that govern the function of the uterus in the important role it plays in reproduction.

1.2.1 THE MENSTRUAL CYCLE

This is a natural, year-round process that occurs in the female reproductive system of some mammals, including humans and a few species of bats and rodents. Sembulingam and Sembulingam (2019) defines the menstrual cycle in humans, as an about 28-day hormone-controlled cyclic event that takes place in a rhythmic fashion during the reproductive period of a woman's life. It usually starts at age 12 to 15 years, known as menarche, and ceases at age 45 to 50 years, known as menopause. Uterine changes that occur during the menstrual cycle are divided into three phases viz: the menstrual phase, the proliferative phase and the secretory phase. According to Liu *et al*., (2020) a normal menstrual cycle in humans consists of these three phases with two peaks of oestrogen secretion and one peak of progesterone secretion. The menstrual phase (Day 1-4): This is the first phase of the uterine cycle in humans. Following ovulation and in the absence of pregnancy, the thickened endometrium is shed and expelled through the vagina along with blood and tissue fluid. This process is called menstruation and may last for 4 to 5 days with the first day of the bleeding regarded as the first day of the menstrual cycle (Sembulingan & Sembulingan, 2019). According to Women in Balance Institute (2023) oestrogen is responsible for growing and maturing the uterine lining. A drop in oestrogen and majorly progesterone levels, from the ovary, is responsible for menstruation. Uterine contractions expel the blood along with the desquamated uterine tissues to the exterior via the vagina. The proliferative phase (Day 5-14): The proliferative phase, also known as the follicular

phase, occurs during the first half of the menstrual cycle, up to day 14 of the menstrual cycle, based on the average duration of 28 days (Thiyagarajan *et al*., 2022). This phase is subdivided into the early, mid-, and late proliferative phases. The early proliferative phase occurs right after menses, usually around day 4 to day 7. The endometrial thickness increases from 2 mm to 5 mm. The mid-proliferative phase usually occurs around day 8 to day 12. The endometrial thickness increases from 5mm to 8mm. The late proliferative phase occurs during the follicular phase (around day 11 to around day 14). The endometrial glands become closely packed and coiled. They also begin to undergo nuclear pseudo-stratification and active mitosis/cell division (Monis & Tetrokalashvili, 2022; Mixhers, 2023). According to Thiyagarajan *et al*., (2022) the main hormone during this phase is oestrogen, specifically 17-beta-estradiol and the purpose of the proliferative phase is to grow the endometrial layer of the uterus and prepare it for the implantation of a fertilized egg. The proliferative phase ends with ovulation, which marks the beginning of the luteal phase. The secretory phase (Day 15-28): This phase occurs from day 14 to day 28 of the menstrual cycle, the period between the day of ovulation and the day when menstruation of next cycle commences. The main hormone during this phase is progesterone, which is stimulated by the luteinizing hormone (LH). The purpose of the secretory phase is to prepare the uterus for implantation of a fertilized egg as the endometrium continues to thicken in response to progesterone. This phase can be subdivided into early and late secretory phases. The early secretory phase occurs from day 14 to day 18 of the menstrual cycle. The endometrial thickness increases from 8 mm to 12 mm. The late secretory phase occurs from day 19 to day 28 of the menstrual cycle. The endometrial thickness remains at 12 mm, and the glands become more coiled and secrete a nutrient-rich fluid to nourish the fertilized egg. The cervix produces less cervical mucus, which becomes thicker and less hospitable to sperm compared to the

cervical fluid in the proliferative phase which appear to be increased and more slippery, with a less acidic vagina pH making it more hospitable for sperm. However, if fertilization does not take place, the corpus luteum, which oversees making progesterone, starts to deteriorate, which causes progesterone levels to fall. This decline in progesterone levels triggers the beginning of menstruation. Menstruation, which signals the start of the subsequent menstrual cycle also signals the end of the secretory phase (Thiyagarajan *et al*., 2022).

1.2.2 THE OESTRUS CYCLE

The female mouse's reproductive cycle, or oestrus, is analogous to the human menstrual cycle. The oestrus cycle in rodents, like mice, lasts an average of 4 to 5 days (Ajayi & Akhigbe, 2020). In the findings of the study above, it was observed that the reproductive period and oestrus cycle of mice commence about the 26^{th} day after birth with the opening of the vagina, which is about ten (10) days before vaginal cornification. According to Cora *et al*., (2015) the apoptosis-mediated vaginal opening in mice is a crucial secondary characteristic in mice, often used as a predictor of puberty. The oestrus cycle comprises of four phases: Proestrus, oestrus, metestrus and diestrus. The visual observation of the vagina is the simplest way to recognize these stages without the need for special equipment. Although it works best when identifying only the proestrus or oestrus phases is required. Vaginal cytology is thought to be the most reliable approach when all four phases need to be recognized since strain to strain variations, especially in coat colour, might make eye inspection difficult to determine the stages (Byers *et al*., 2012). A comprehensive summary of these stages as studied by Ajayi & Akhigbe (2020) is provided: The proestrus phase (cycle length in hours: < 24): The luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion increases during this period, which corresponds to the human follicular stage. It is the period leading up to an animal going into heat. The vagina

appears gaping and the tissues are wet and reddish pink at this time. There is a low to moderate relative cell density with most of the cells being tiny nucleated epithelial cells following vaginal cytology or smear evaluation. The cuboidal to columnar transition of uterine epithelial cells occurs with little to no cell degeneration and an increase in cell mitosis. The oestrus phase (12 – 48 hours): The term “oestrus” was originally used by British physiologist, Heape (Heape, 1990), a Latin translation of the Greek word, “oistros” meaning “sexual season”. The peak in FSH concentration with an associated rapid decline in oestradiol levels correlates to ovulation (in man) and oestrus phase (in mice). The vagina seems to be like that observed at proestrus, but with the tissues lighter pink and less wet. There is also minimal to high amount of anucleate keratinized (cornified) epithelial cells density at this stage. There is also the appearance of notable degradation of uterine epithelial cells and glands with reduction of mitotic activity and minimal dilatation of the uterus. The metestrus phase (8 – 48 hours): Metestrus is like the human early secretory phase of the reproductive cycle with high levels of progesterone. It is a brief time period that takes place in the absence of conception. The tissues of the vagina seem pallid and dry. White cellular debris may line the inner walls or partially fill the vagina. Neutrophils and certain cornified epithelial cells may have a moderate to high relative cell density on a slide smear. In the uterus, endometrial epithelial cells show continued degeneration and the return of mitotic activity. Metestrus will begin if the cycle is not broken by pregnancy, pseudo-pregnancy, or anoestrus at the oestrus phase. The dioestrus phase (48 – 72 hours): This phase is also comparable to the late secretory phase of the human reproductive cycle, with high levels of progesterone. It is the brief time of rest during the mating season before the start of the following proestrus. The vagina opening of some mice may appear moist, too small and even closed with no signs of swelling. Neutrophils and nucleated epithelial cells are present in low to moderate

relative abundance in the vagina at this stage. The uterus has a small, avascular, slit like lumen, that is bordered with low columnar epithelial cells that are mostly cuboidal in shape. The dioestrus stage, which lasts for more than two days before the cycle is repeated, is the longest of the stages. The anoestrus phase is the non-breeding period when reproductive organs are dormant. Among the other changes in the characteristics of the uterine morphology during the four stages listed above, it is worthy of note that rodents do not naturally menstruate, with the only exception to date being the Egyptian spiny mouse, *Acomys cahirinus* (Bellofiore *et al*., 2017; Bellofiore *et al*., 2018; Bellofiore *et al.*, 2021). In fact, the endometrium, in rodents like mice and most mammals, instead of being shed with blood flow from the vagina (as in true or overt menstruation), is broken down and totally reabsorbed without vaginal discharge at the end of its reproductive cycle. This is regarded as covert menstruation (Catalini & Fedder, 2020). Despite being occasionally challenged as a model for reproductive research (Neuber & Powers, 2000; Carter, 2020), mouse models have long been a crucial investigative tool in reproductive biology and medicine research as experimental models for human reproductive pathophysiology. Some of the many advantages in using the mouse as the choice of animal model in this research include its small size and availability, with relatively low maintenance costs and short reproductive cycle as well as its physiological similarities to the human uterus allowing for an efficient study.

1.3 UTERINE CONTRACTIONS: AN OVERVIEW

Uterine contractions are muscular uterine smooth muscle contractions that take place throughout the menstrual cycle and during labour. They happen throughout the menstrual cycle in women who are not pregnant and throughout pregnancy as well (Wikipedia, 2023). According to Aguilar & Mitchell, (2010) the four crucial characteristics for defining uterine contractions that change under various physiological or pathological conditions are frequency, amplitude, duration and

direction of propagation. Overall, uterine contractile activity plays an important role in many and varied reproductive functions, including:

Sperm, embryo transport & implantation: Uterine contractions help to move sperm and embryos through the female reproductive tract. These contractions also aid in the implantation of the embryo into the endometrium (Aguilar & Mitchell, 2010). Menstruation: Uterine contractions are necessary for emptying or discharging the endometrial womb lining. The pattern of contractile myometrial activity, for example, can be significantly influenced by changes in female steroid hormones during menstruation (Bafor *et al*, 2017). Labour and delivery: Uterine contractions play a crucial role in the process of natural childbirth, which entails the ejection of the foetus and its placenta from the uterus to the outside world (Riemer & Heymann, 1998). Reproductive disorders: Understanding the physiological pathways and molecular mechanisms of uterine contractility is important for the development of treatments for pathological conditions. Abnormal uterine contractility might underline disorders such as infertility and spontaneous miscarriage (Sajadi *et al*., 2018), implantation failure, preterm births and the need for Caesarean section delivery (Aguilar & Mitchell, 2010), dysmenorrhea and endometriosis (Huang *et al.*, 2017). In general, the non-pregnant uterus's contractile activity appears to differ significantly from that in pregnancy. In the non-pregnant state, uterine contractions occur throughout the menstrual cycle, also termed endometrial or contractile waves, and seem to only affect the sub-endometrial layer of the myometrium. The frequency of contractions rises as ovulation approaches and decreases during the luteal phase, presumably to aid implantation. If implantation is unsuccessful, the frequency of contractions remains low but at menstruation the intensity increases again (Aguilar & Mitchell, 2010). These intense contractions are sometimes termed, "menstrual cramps" (Stoppler & Shiel, 2023). In contrast, during pregnancy, the uterus

undergoes significant modifications that facilitate uterine adaptation to the growing foetus. The myometrium is recognized for having rhythmic contractions that cause Braxton Hicks contractions during pregnancy, false labour contractions close to the time of birth, and actual labour around the end of the third trimester (Garfield & Maner, 2007). Throughout the early stages of pregnancy, the myometrium has been described as quiescent, with the cervix rigid and closed. The uterine contractions are normally of irregular pattern with weak intensity to retain the conceptus and enable the embryo to grow. The uterus however transforms during labour to a very powerful active organ to expel the foetus and placenta, with the contractions going from episodic and uncoordinated to highly coordinated and rhythmic (Otaibi, 2014).

1.4 REGULATION OF UTERINE CONTRACTIONS IN PREGNANT UTERUS

The regulation of uterine contractions typically involves hormonal, neural, inflammatory mediators and mechanical factors.

1.4.1 HORMONE REGULATION

Numerous studies have shown oestrogen, progesterone and oxytocin as the three primary hormones involved in uterine contractions. Oxytocin, a hormone involved in uterine contractions, affects calcium regulation in the myometrial cells. It inhibits the calcium/ATPase of the myometrial cell membrane which pumps calcium from the inside of the cell to the extracellular space, leading to decreased calcium efflux from the cell and increases the influx of calcium. Oxytocin also triggers the release of calcium from the sarcoplasmic reticulum. Ilicic *et al.* (2017) study suggests the oestrogen to progesterone ratio during late pregnancy increases the number of oxytocin receptors on the uterus, contributing to enhanced oxytocin response. Oxytocin is produced by the hypothalamus and released by the posterior pituitary gland into the blood stream which delivers it to oxytocin receptors on myometrial cells. These receptors are rhodopsin class

1 G proteins that couple with phospholipase C (PLC), activating Inositol Triphosphate (IP_3) and Diacylglycerol (DAG) signalling. Activated IP_3 mobilizes calcium from the sarcoplasmic reticulum, which then binds to myosin light chain kinases resulting in smooth muscle contraction. Through its positive feedback loop along the uterine epithelial lining, oxytocin also plays a crucial role that further catalyses the onset of labour (McEvoy & Sabir, 2022). Progesterone, another hormone, plays a crucial role in maintaining pregnancy by decreasing the permeability of uterine cells to calcium, sodium and potassium ions. It also influences intracellular calcium binding, reducing the availability of calcium for the calcium-myosin light chain kinase (MLCK) system. Also, progesterone increases cyclic adenosine monophosphate (c-AMP) synthesis, which further contributes to uterine relaxation. It is vital for the maintenance of pregnancy as it causes uterine relaxation during early pregnancy. When functional progesterone is removed, the oestrogen-progesterone ratio rises, which raises prostaglandin concentration and starts labour (Nadeem *et al*, 2016). Oestrogen, also a hormone, stimulates the production of prostaglandins F and E which stimulate uterine contractions. According to Weiss (2000) it also increases connexin 43 synthesis and gap junction formation in the myometrium, allowing for coordinated uterine contraction. His study also suggests that oestriol concentrations in serum and saliva increase during the last 4 to 6 weeks of pregnancy, and there seemed to be a four-week advancement in the higher levels of salivary oestriol in women who will deliver preterm. Weiss therefore suggests a salivary oestrogen screening in pregnant women with the potential of preterm labour risk.

1.4.2 NEURAL FACTORS

Uterine contractions are part of the process of natural childbirth and are also controlled by neural regulation. The uterus must change from a dormant structure with dys-synchronic contractions to

an active, coordinated-contracting organ during parturition. This requires gap junction formation between the myometrial cells to allow for transmission of the contractile signals (Weiss, 2000). Several types of neurons play a role in uterine contractions *viz*; cholinergic neurons found in the pelvic autonomic ganglia and uterus produce acetylcholine (Ach), which stimulates contraction of the uterus and dilates the uterine arterial supply (Papka *et al*., 1999). Uterine contractions trigger both oxytocin and vasopressin neurons in the supraoptic nucleus via a noradrenergic route (Douglas et al., 2001). Sensory, sympathetic, and parasympathetic fibres are present in the uterus and cervix and in fact, there is scientific evidence to imply a link among oestrogen, uterine cervix-related sensory neurons of the lumbosacral (L6-S1) dorsal root ganglia and parturition (Papka & Mowa, 2004).

1.4.3 INFLAMMATORY MEDIATORS

Inflammatory markers, most notably prostaglandins are labour mediators. The two most studied prostaglandins (PGs) closely associated with uterine contractions are PGE1 and PGE2. They induce myometrial contractility, possibly operating as calcium ionophores leading to an increase in intracellular calcium. PGE2 stimulates EP1 and EP3 receptors on myometrial cells and during labour, activates interleukin-8 (IL-8) and tissue necrosis factor-alpha (TNF-alpha). This action activates collagenases and Matrix Metalloproteinases (MMPs) causing the “ripening” of the cervix. PGF2-alpha is expected to lower progesterone levels and, separately, improve uterine contractility by activating smooth muscle cells. After the birth of the foetus, uterine contractions are also influenced by PGs. The placenta secretes PGs during stage three of labour, which causes its dissociation from the endometrial cavity. Contractions during this time period additionally minimize postpartum bleeding (McEvoy & Sabir, 2022).

1.4.4 MECHANICAL FACTORS

From various studies done previously, factors such as foetal movement, uterine distention, pressure of the foetus against the cervix, has been shown to stimulate uterine contractions. These are termed, “mechanical factors” and can affect the strength and frequency of uterine contractions via mechanisms such as triggering the release of prostaglandins or oxytocin which cause uterine contractions. Mechanotransduction is an accepted phenomenon of the myometrium and this mechanism plays a role in coordinating uterine contractions in human labour. In these processes, mechanical impulses are transformed into biochemical signals that control uterine contraction (Rozin, 1957; Wood, 1964; Cochran & Gao, 2015; Young, 2016).

1.5 PHARMACOLOGY OF UTERINE CONTRACTILITY: TOCOLYTICS AND OXYTOCICS

1.5.1 TOCOLYTICS

These are agents used to inhibit uterine contractions and prevent preterm labour. A brief summary of Arrowsmith *et al*. (2010) review on these agents is provided below:

1. Progesterone: A steroid hormone that sustains the pregnant state and supports uterine quiescence. Mechanism of action (MOA): Its interaction with the intracellular nuclear progesterone receptor is believed to be the primary mechanism of action. It inhibits the action of phosphodiesterase (PDE). *In vitro* progesterone suppresses both spontaneous and oxytocin-induced contractions and decouples the excitation-contraction pathway. It inhibits calcium entry and sarcoplasmic reticulum (SR) calcium release, in addition to producing membrane hyperpolarization via activation of potassium channels. Clinical uses include preventing preterm delivery while synthetic progesterone supplementation;

17-alpha-hydroxyprogesterone, has been demonstrated to be effective in preventing preterm labour in singleton deliveries but not in multiple pregnancies.

2. Magnesium sulphate: An antagonist to calcium and a cofactor for several processes. As a result of its competition with calcium, it influences several intracellular processes. MOA: Relaxes smooth muscles via unknown processes. It has been demonstrated, nevertheless, that it affects both extra and intra cellularly by reducing calcium entry and possibly blocking SR calcium release. Antagonism between magnesium and calcium results in a dose dependent reduction in intracellular calcium as well as the amplitude in both spontaneous and oxytocin induced contractions in pregnant human myometrium. Clinical uses: Magnesium has long been utilized as a tocolytic in obstetrics, and more recently, it has been used to prevent and treat pre-eclampsia.

3. Calcium Channel Blockers (CCBs): Nifedipine is a well-known example. These medications were designed to inhibit voltage gated calcium channels and were first introduced to treat hypertension. They stop the myometrial cells' normal increase in intracellular calcium. MOA: Blocking of calcium channels prevents the entry of calcium and the distribution of the action potentials necessary for the myometrium to contract in coordination. *In vitro*, calcium channel blockers decrease and subsequently cease the contraction of the myometrium in both preterm and term women. Clinical uses: compared to the other tocolytic agents, CCBs considerably postpone birth for up to seven days when taken prior to week 34 of pregnancy. Mibefradil, T-type channel blocker, has been shown to cause a significantly lessen the contraction of the human myometrium during pregnancy and prevent calcium release from the SR. On the other hand, (Asokan *et al*.,

2002) had no impact on oxytocin-induced contraction in Ca^{2+} free physiological salt solution (PSS) on uterine strips from non-pregnant rats.

4. Oxytocin antagonists: Antagonists to the oxytocin receptor. They bind to oxytocin receptors competitively and prevents the effects of oxytocin. MOA: by preventing oxytocin binding, stimulation of uterine contractions would then be inhibited. Atosiban, an oxytocin derivative is a competitive inhibitor of oxytocin receptors as well as the vasopressin (V1a) receptor. Barusiban, on the other hand, has a higher affinity for the OTR and a higher potency than Atosiban, without its vasopressin receptor antagonism side effects. Although, both drugs have been studied in clinical trials for their effects on uterine contractions and their potential use in invitro fertilization (IVF) treatment and prevention of preterm births, only Atosiban has been licensed and approved for clinical use.

5. Beta adrenergic receptor agonists: These are drugs developed to stimulate β-2 adrenergic receptors. They include terbutaline, salbutamol. They relax smooth muscles including the myometrium. MOA: as agonists at β-2 receptors, they activate adenylyl cyclase pathway, thereby increasing cyclic Adenosine Monophosphate (c-AMP) and activating Phosphokinase A (PKA). Clinical uses: there is a large body of evidence supporting the use of β-2 adrenergic receptor agonists for the treatment of preterm labour. The extent of the tocolysis produced however is limited by its possible extreme side effects profile as a result of the widespread distribution of β-2 adrenergic receptors in the body as well as desensitization concerns.

6. Prostaglandin synthesis inhibitors: Drugs that prevent the synthesis of prostaglandins. They include cyclo-oxygenase inhibitors, NSAIDs such as Indomethacin. MOA: They

decrease myometrial prostaglandin output by inhibiting prostaglandin production pathway, thereby reducing myometrial contractions. NSAIDs inhibit COX-1 and COX-2 and were first used as tocolytics because by preventing the release of prostaglandins and cytokines, they could delay preterm delivery when given at the onset of preterm labour. However, NSAIDs have several other risks associated with their use. Another approach is via direct inhibition of prostaglandin receptors. THG113 is a novel, selective, non-competitive Prostaglandin F (FP) antagonist that has been shown to significantly delay preterm delivery. Its mechanism of action is to block the activity of prostaglandin F-2 α receptor, which is involved in the regulation of uterine contractions thereby delaying premature delivery and reducing uterine activity.

7. Nitric oxide donors: Producers of the short lived, metabolically active gas, nitric oxide. A typical example here is glyceryl trinitrate or sodium nitroprusside. They relax smooth muscle particularly of blood vessels. MOA: Nitric oxide when released increases levels of cyclic guanosine monophosphate (c-GMP) and phosphokinase G (PKG) and can thereby affect several pathways associated with relaxation. Nitric oxide is formed from L-arginine by the action of nitric oxide synthetase. Clinical uses: nitric oxide donors have been employed for cervical ripening, labour induction and tocolysis in female reproductive health.

1.5.2 OXYTOCICS (UTEROTONINS)

Oxytocics are used to stimulate uterine contractions and induce labour.

1. Oxytocin: The most common oxytocic drug. It is a nona-peptide hormone released from the posterior pituitary gland. Oxytocin stimulates contraction of the myometrium, and myo-epithelium of the mammary ducts (for milk ejection) and influencing maternal

behaviour. MOA: Activation of oxytocin receptors cause phospholipase C activation, which hydrolyses phosphatidylinositol bisphosphate (PIP2) leading to the formation of two secondary messengers, IP3 and DAG. IP3 stimulates calcium release from the SR. Oxytocin also inhibits calcium efflux mechanisms and myosin light chain proteins, slowing relaxation and enhancing contraction force. In pregnancy, OTRs increase in number which is thought in part to underlie the increased sensitivity of the myometrium to oxytocin at term. Clinical uses: it is widely used in its synthetic forms, for labour augmentation and induction. Also, postpartum haemorrhage and impaired milk ejection.

2. Ergot alkaloids: Ergometrine (Ergonovine or natural ergot) and Methyl ergometrine (or methylergonovine) are classical examples. MOA: Ergonovine directly stimulates the uterine muscle to increase force and frequency of contractions. It also produces arterial vasoconstriction by stimulation of the α-adrenergic and serotonin receptors alongside the inhibition of endothelial derived relaxation factor release. Ergot alkaloids induce tetanic contraction of uterus without relaxation in between. It causes contractions of the uterus as a whole unit. That is, both the fundus and cervix contract, thereby tending to compress rather than to expel the foetus. Clinical uses: Postpartum haemorrhage (third stage of labour only)

3. Prostaglandins: They are bioactive lipids produced from arachidonic acid. They are crucial during the entire pregnancy. Through the enhancement of contractions and "ripening" of the cervix, they have been demonstrated in both the onset and maintenance of term labour as well as in certain premature labours. MOA: They act as signalling messengers that mediate their actions by interacting with G-protein coupled receptors, which increases intracellular calcium and enhances greater actin-myosin interaction and

contraction. Since each PG has a unique receptor, this facilitates the activation of several intracellular signalling cascades. For instance, stimulation of the FP, EP1, EP3 and TP receptors causes a contractile response whereas activation of the EP2, EP4, IP and DP receptors causes calming effects. PGF2-α and PGE predominate in the myometrium. Clinical uses: For many years, PGs have been used therapeutically for abortion, labour induction, and cervical preparation before labour induction. Misoprostol and gameprost, synthetic equivalents of PG, are used for medical termination. The drug carboprost has been used to promote labour contractions and lessen postpartum bleeding. Since dinoprostone is safer than misoprostol, it is widely used for cervical ripening.

1.6 RUTIN HYDRATE: OVERVIEW, PROPERTIES AND ETIOLOGY

Rutin is a flavonoid glycoside that combines the flavanol, "quercetin" and the disaccharide "rutinose". Its name originates from the plant, *Ruta graveolens* (Wikipedia, 2023). Its properties include:

Name: Rutin.

Other names: Rutoside, Vitamin P, Quercetin-3-O-Rutinoside, Sophorin.

IUPAC Name: 3',4'5,7-tetrahydroxy-3-[α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranosyloxy] flavone.

Chemical formula: $C_{27}H_{30}O_{16}$

Molar mass: $610.521 g.mol^{-1}$

Appearance: Solid

Melting point: $242^{O}c$

Source: Citrus, apples, buckwheat, asparagus, tea and passionflower are just a few of the many plants that contain rutin hydrate (Ganeshpurkar & Saluja, 2017; Patel & Patel, 2019). Citrus leaves contain rutin hydrate in quantities of 7 and 11 g/kg in orange and lime trees, respectively. Citrus fruit peels contain 34 to 49 mg/g of flavonoids represented as rutin hydrate equivalents. (Wikipedia, 2023).

A crucial phytochemical required for optimal health is rutin hydrate. Since rutin, one of the key components of apples, has shown a variety of pharmacological and biological effects, the proverb "an apple a day, keeps the doctor away" seems to apply to rutin. According to Ganeshpurkar & Saluja (2017), rutin has showed a number of pharmacological effects, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective actions. Already commercially available as an orally administered supplement to support wound healing, Pivec *et al*., (2019) study describes rutin hydrate as best known for its antioxidant, wound healing and anti-inflammatory properties. Recent research on rutin hydrate has mainly focused on its use in cosmetic and pharmaceutical products.

1.6.1 POTENTIAL BENEFITS OF RUTIN IN REPRODUCTIVE AND MENSTRUAL HEALTH

There is limited scientific evidence on the potential benefits of rutin in reproductive and menstrual health. However, some studies suggest that rutin hydrate may have positive effects in this area. Some potential benefits of rutin already studied for its use in reproductive health include: Rutin, because of its antioxidant property, partially reverses some of the cisplatin-related toxicity effects on testicular tissue such as testicular damage and sperm count. Hence, it is suggested that rutin may be used as a potent neuroprotective therapeutic agent when combined

with cisplatin to reduce its potential neurotoxic side effects (Jahan *et al*., 2018). There is evidence for the potential ameliorative effects of rutin against clinical and biochemical features of polycystic ovarian syndrome (Jahan *et al*., 2016). Rutin possessing antioxidant and anti-inflammatory effects coupled with its common use for capillary fragility, suggests it may generally be beneficial for menstrual health (Ganeshpurkar & Saluja, 2017).

1.7 RATIONALE FOR THIS STUDY

Smooth muscle cells are generally responsible for regulating contractions in various organs, including the uterus, vasculature, respiratory, digestive and urinary tracts. Smooth muscle contraction is initiated by calcium-regulated phosphorylation of myosin, which causes increased tension along the entire chains of tensile structures, ultimately resulting in contraction of the entire smooth muscle tissue (Wikipedia, 2023). Rutin is a flavonoid that has been studied for its effects on the contraction of isolated smooth muscles. It was found to inhibit the contraction of isolated rat aorta smooth muscle induced by high potassium and phenylephrine (Ajay *et al*., 2003). It has been suggested to modulate Ca^{2+} uptake significantly in rat pancreatic islets, by opening L-VDCCs, altering intracellular Ca^{2+}, PLC and PKC signalling pathways, while exhibiting KATP channel independent mechanisms, suggesting it to be a potential insulin secretagogue and to modulate glucose homeostasis (Kappel *et al*., 2013). Rutin has also been shown to induce a concentration-dependent relaxation of rat smooth muscle possibly via nitric oxide-guanylyl cyclase pathway for aorta and showed suppression of contractions by likely anticholinergic activity for ileum and trachea tissues (Shekha & Al-Habib, 2013). Arefani (2018) study further suggested that the relaxant effect of *L. Album* extract on trachea tissues might have been caused by the presence of various phenolic compounds (including rutin) found in the extract. More recently, Talebi *et al*., (2021) study showed that rutin improves endometriosis by

apoptosis and antioxidant mechanisms. These findings suggest that rutin may have potential therapeutic benefits for conditions that involve smooth muscle contraction, such as in circulatory, respiratory health, gastrointestinal and possibly uterine disorders. The rationale for studying the effects of rutin on pregnant uterine smooth muscle contraction using isolated mouse uterus is to understand how rutin affects the contractility of uterine smooth muscle cells as these cells play a critical role responsible for regulating uterine contractions during pregnancy and labour. Previous studies have investigated the effects of various compounds on uterine smooth muscle contraction using isolated rat and mouse uterus, including nitrendipine (Milovanovic *et al*., 1989), ligustilide (Junrong *et al.,* 2006), thrombin (Fitzgibbon *et al*., 2009), and caffeic acid (de Alencar Silva *et al*., 2020). Furthermore, flavonoids like *Pimpinella anisum* extract was found to relax pregnant uterine contractions possibly by blocking calcium entry via L-type calcium channels in term-pregnant rats (Alotaibi, 2020). Adlay hull extracts increased the expressions of COX-2, ERK1/2, and PKC-alpha receptors, all of which are involved in uterine contractions in rats. (Hsia Shih-Min *et al*., 2008). Resveratrol, a grape polyphenol, was found to reduce oxytocin-induced uterine contractions in rats (Hsia Shih-Min *et al*., 2011). *Matricaria chamomilla* flavonoids extract was found to have spasmolytic actions on isolated rat uterus (Sadraei *et al*., 2020). These studies suggest that different flavonoid substances can affect uterine contractions in animal models, but there is limited specific information on the effect of rutin on the contraction of the pregnant uterus. Generally, abnormal uterine contractions during pregnancy can cause various complications for both the mother and the foetus. Some effects, among others, include: Preterm birth: Dysfunctional uterine contractions can cause preterm labour and delivery, which may result in complications such as respiratory distress syndrome, intraventricular haemorrhage, and necrotizing enterocolitis (Malik *et al*., 2021). Dystocia: Abnormal uterine contractions can cause

dystocia, which is a difficult or prolonged labour that can lead to foetal distress, maternal exhaustion and the need for instrumental delivery or Caesarean section (Gulumser & Yassa, 2022). Uterine atony: Weak or absent uterine contractions after delivery can cause uterine atony, which can lead to postpartum haemorrhage and other complications (Malik *et al*., 2021).

Overall, abnormal uterine contractions during pregnancy can cause various complications and require careful management to ensure the safety of both the mother and the foetus. Therefore, in the search for new targets and complementary drug therapies for managing the effects of abnormal uterine contractions in pregnancy, this study sets out to investigate rutin hydrate's activity on uterine contractions in pregnant mouse models and additionally attempt to identify its possible mechanism of action.

1.8 RESEARCH QUESTIONS, STUDY HYPOTHESIS, AIMS AND OBJECTIVES

1.8.1 RESEARCH QUESTION

The following questions will guide our research: Does rutin, a flavonoid compound, affect the contraction of isolated pregnant mice uterus in a dose-dependent manner? What is the possible mechanism of action of rutin on uterine contractility?

1.8.2 STUDY HYPOTHESIS

Based on the existing literature and preliminary observations as studied above, we propose the following hypothesis. Rutin induces a dose-dependent relaxation of spontaneous, oxytocin-induced, high KCL-induced and oxytocin-induced contractions in a zero-calcium medium by modulating calcium activity in myometrial cells.

1.8.3 AIM OF THE STUDY

The aim of this study is to investigate the effect of rutin hydrate on spontaneous and agonist-induced contractions of isolated pregnant mice uterus.

1.8.4 OBJECTIVES OF THE STUDY

The objectives of this study were to determine:

1. The effect of rutin hydrate on spontaneous uterine contractions in pregnant mice uterus.
2. The effect of rutin hydrate on oxytocin-induced uterine contractions in pregnant mice uterus.
3. The effect of rutin hydrate on high KCL-induced uterine contractions in pregnant mice uterus.
4. The effect of rutin hydrate on oxytocin-induced pregnant mice uterine contractions in a zero-calcium medium.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 LABORATORY MATERIALS

Laboratory materials used in this study include; micropipette (Microflux) 0 – 1000 uL, sample bottles, beakers (50 mL, 250 mL and 500 mL), Pasteur pipettes, syringes (2 mL, 5 mL, 20 mL), needles, white thread, masking tape, permanent markers (red, blue, green and black), dissecting instruments, glass stirrer, brushes, disposable gloves, plastic cages and aerated lids, spatula, measuring cylinders (100 mL and 250 mL), microscope, glass slides, distilled water, porcelain dish, pestle, hot plate/oven, organ and water bath, digital weighing balance, Uninterrupted Power Supply (UPS), laptop with the labchart software (ADInstruments), 7003E-isometric force transducer (Panlab ADInstruments, Spain), PowerLab 2/26 Model ML826 data acquisition/recording unit (ADInstruments, Australia) and the GraphPad Prism v. 8.1 (San Diego,CA,USA) for analysis.

DRUGS, CHEMICALS AND REAGENTS

Rutin Hydrate (Sigma-aldrich), Dimethyl sulphate solution (DMSO4) and Acacia gum of high analytical grade were utilized in this study. Physiological saline solution (PSS), Ringer's Locke was prepared with the following composition (mM/L): NaCL 154.00, KCL 5.63, D-Glucose 2.78, $NaHCO_3$ 5.95 (from Sigma Aldrich, UK), CaCL.2H20 2.05 (XL China). Others include; Methanol (Pharmatrends, Nigeria), Ethylenediaminetetraacetic acid (EDTA) (Guangdong GuanghuaSci-Tech Co. Ltd China), Normal Saline (BioFLEX; Biomedical Nigeria Ltd), Oxytocin (Roche Pharmaceutical Ltd), Gentian Violet dye (Nomagbon Pharmaceuticals Ltd, Nigeria).

2.2: ANIMALS

This study was conducted using healthy pregnant female albino mice (30-42g). These mice were procured through the animal unit of the Faculty of Pharmacy, housed and maintained in the same facility within the University of Benin, Nigeria, situated at the Department of Pharmacology and Toxicology. For mating conditions to achieve pregnancy, virgin female albino mice were paired with a male mouse of the same strain overnight at the ratio of 2:1. Gestation day 0 was defined by the presence of vaginal plug and animals on gestation day 18 were used for this study. The mice were allowed adaptation for two weeks before the commencement of the study. They were maintained under a natural light/dark cycle at room temperature. They were provided with a standard diet of rodent pellet feed (Top feeds limited, Ibadan, Nigeria) and had continuous access to clean tap water *ad libitum*. The study received ethical approval from the Faculty of Pharmacy Ethics Committee at the University of Benin. The care and treatment of the animals strictly adhered to the guidelines outlined in the 'Guide for the Care and Use of Laboratory Animals' and the 'Public Health Service Policy on Humane Care and Use of Laboratory Animals' (National Research Council, 2010, Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2015).

2.3 EXPERIMENTAL PROTOCOL

2.3.1 UTERINE TISSUE PREPARATION

The healthy pregnant mice were time-mated and humane euthanasia was carried out on the eighteenth (18^{th}) day of gestation through cervical dislocation. The abdomen was opened, uterine horns were promptly extracted, cleaned of connective tissues, adhering fats, and placed in a petri dish filled with aerated and warmed PSS. The foetuses and their placenta were carefully detached from the uterus and the uterine tissues were then dissected into uterine segments

measuring approximately 1 – 2 mm in length. These segments were subsequently mounted in an aerated organ bath containing 10 mL of Ringers Locke solution, maintained at a temperature of 37ºC. The composition of this physiological salt solution per Mm/L was as follows: CaCl2.2H2O = 2.05, D-Glucose = 2.78, KCl = 5.63, NaHCO3 = 5.95, and NaCl = 154.00, as previously described. (Bafor *et al*., 2015; Bafor *et al*., 2019). All uterine segments obtained were vertically mounted by tying with surgical thread at both ends (making a loop on one end) using a sterile needle and was immersed within the 10 mL organ bath as explained above. The loop was attached to a tissue holder and the long threaded opposite end was attached was connected to a 7003E-isometric force transducer (Panlab ADInstruments, Spain), linked to bridge amplifiers further connected to a PowerLab data acquisition system consisting of a recording unit for recording and showing changes in the force and frequency of the contractions (PowerLab 2/26 Model ML826 ADInstruments, Australia). LabChart 7 reader software (v.8.0, ADInstruments, North America, USA) was further used to note the measurement. Proper conditions were ensured by allowing the uterine tissue to equilibrate under suitable resting tension of 0.5 g in the PSS until regular rhythmic contractions were observed before the addition of drugs (Uchendu & Bafor, 2023).

2.3.2 EXPERIMENT ON SPONTANEOUS UTERINE CONTRACTILITY IN PREGNANT MICE

Rutin hydrate (0.03 – 25 mg/mL RT), was cumulatively introduced to the isolated pregnant uterine tissues after obtaining regular spontaneous uterine contractions, which served as control. Following the addition of each concentration, a 5-minute contact time was observed, as described in Bafor *et al*.'s studies (2019).

2.3.3 EXPERIMENT ON OXYTOCIN-INDUCED UTERINE CONTRACTION

Oxytocin (OT) at a concentration of 11.62 nM was introduced to the pregnant uterine tissues for a ten-minute period to pre-stimulate it then washed with fresh PSS and allowed to recover regular contractions. Thereafter, the uterine tissue was pre-contracted again with same oxytocin dose for 10 minutes. Without a washout step, single dose of RT (25 mg/ml) was added in the continued presence of OT. After this dual exposure, the tissues underwent washing and a subsequent recovery period. Changes in amplitude and frequency were measured and subjected to analysis. (Bafor *et al*., 2020; Uchendu & Bafor, 2023)

2.3.4 EXPERIMENT ON HIGH POTASSIUM CHLORIDE (KCL)-INDUCED UTERINE CONTRACTION

Potassium chloride (KCl) at a concentration of 80 mM was introduced into the organ bath for a 10-minute duration to pre-contract the tissues. Subsequently, washing was carried out and the tissue was allowed to recover. Again, the tissue was pre-contracted with the same dose of high KCL for 10-minutes and without a washout step, single dose of RT (25 mg/ml) was added in the continuous presence of high KCL (80 mM). (Uchendu & Bafor, 2023).

2.3.5 EXPERIMENT ON OXYTOCIN-INDUCED UTERINE CONTRACTION IN A CALCIUM (CA)-FREE MEDIUM

In this experiment, a calcium-free PSS containing ethylenediaminetetraacetic acid (EDTA) was used to assess the effect of RT on OT-induced contraction. After an initial thirty-minute equilibrium period of spontaneous uterine tissues in the normal Ringer's Locke PSS, the transition to the zero-calcium PSS solution containing 0.1 mM EDTA was made. The tissue was then equilibrated in the calcium-free solution for about three minutes while ensuring contractions were not completely eliminated during this phase to facilitate measurement. Subsequently,

without draining the Ca^{2+}-free PSS, OT (11.6 nM) was introduced and subsequently, RT (25 mg/ml) was added in the continuous presence of OT (Uchendu & Bafor, 2023).

CHAPTER THREE

3.0 RESULTS

3.1 EFFECT OF RUTIN HYDRATE ON SPONTANEOUS UTERINE CONTRACTION

Rutin hydrate (RT) cumulative additions led to dose-dependent relaxation in spontaneous pregnant uterine contractions (Figure 3.1a). In the presence of RT, both the frequency and the amplitude of the contractions progressively reduced until contractions were eliminated (Figure 3.1b). The Emax of RT for amplitude and frequency in the pregnant mouse tissues was estimated. There was immediate recovery of spontaneous uterine contractions after the washout of RT with fresh PSS.

3.2 EFFECT OF RUTIN HYDRATE ON OXYTOCIN-INDUCED PREGNANT UTERINE CONTRACTIONS

Oxytocin stimulated an increased amplitude and frequency of uterine contractions (Figure 3.2a). However, RT in the simultaneous presence of OT did not show any significant observed relaxation in the strength or regularity of these contractions. It was clearly noted on analysis of the frequency (B) and amplitude (C), that RT did not exhibit significant inhibition of these parameters in the pregnant uterus (Figure 3.2b).

3.3 EFFECT OF RUTIN HYDRATE ON HIGH KCL-INDUCED PREGNANT UTERINE CONTRACTIONS

High KCL (80 mM) caused a rapid and sustained increase in the force of pregnant uterine contractions (Figure 3.3a). RT in the continuous presence of high KCL however did not substantially relax high KCL-induced uterine contractions. This was evident as the force of contractions (B) induced by KCL (80 mM) remained largely undiminished (Figure 3.3b).

3.4 EFFECT OF RUTIN HYDRATE ON OXYTOCIN-INDUCED UTERINE CONTRACTIONS IN A CALCIUM FREE MEDIUM

The addition of OT to the uterine tissue mounted in a zero-calcium PSS, containing the calcium chelating agent, EDTA, slightly increased the spontaneous contractions of the pregnant uterine tissue (Figure 3.4a). In the presence of RT however, oxytocin-induced contractions (11.62 nM) in a calcium-free medium showed no significant inhibition. RT did not induce notable relaxation in the frequency (B) and amplitude (C) of these contractions in the presence of zero calcium (Figure 3.4b).

A

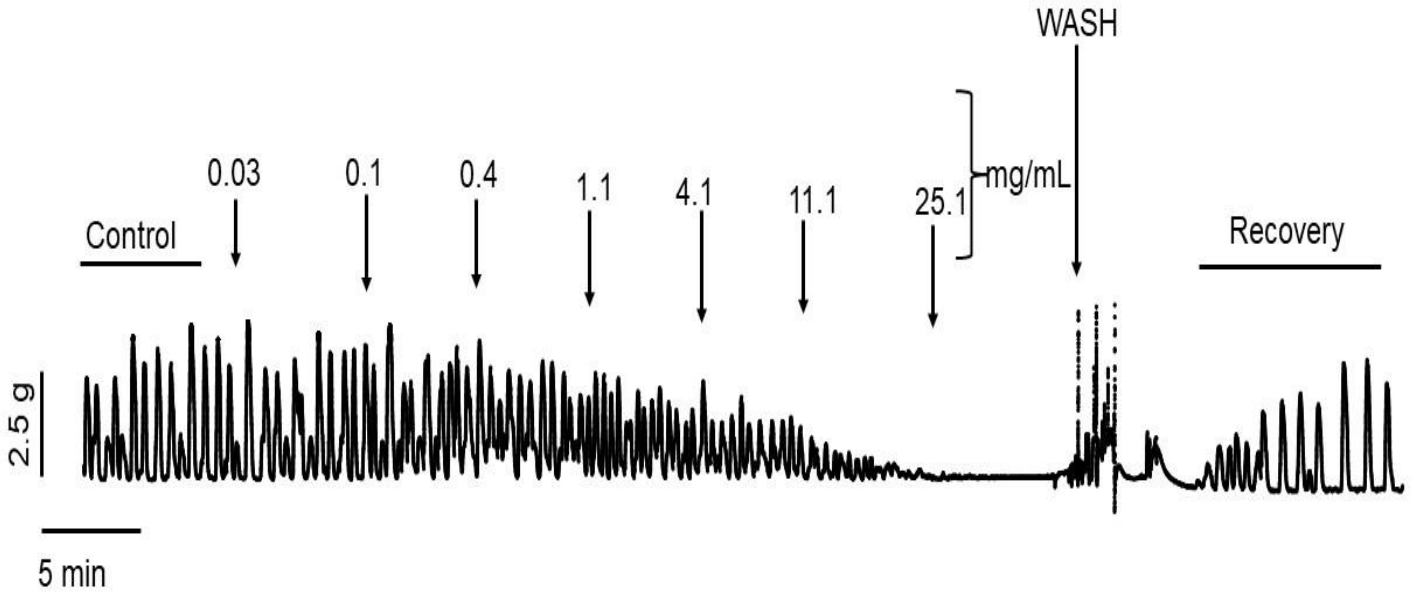
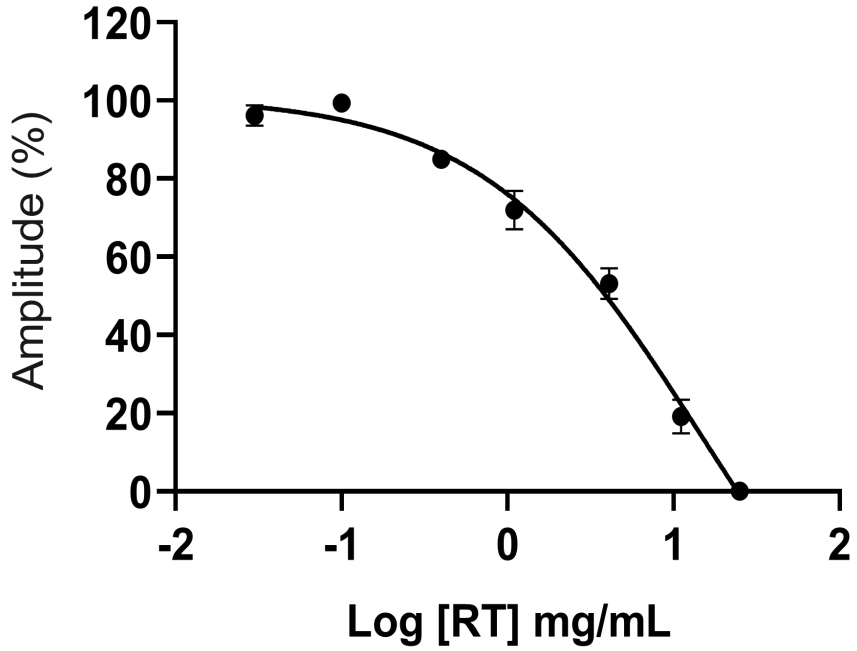
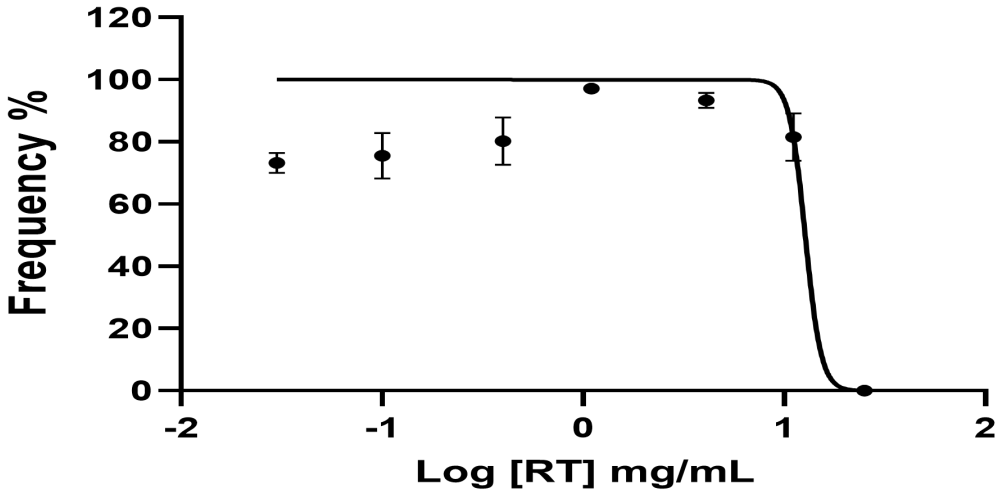


Figure 3.1a Original representative recording showing the effect of RT on spontaneous contractions in an isolated pregnant mouse uterus. RT = Rutin hydrate. n = 5 animals.



B



C

Figure 3.1b Concentration response curve showing the effects of RT on the frequency and amplitude of spontaneous contractions in isolated pregnant mouse uterus. RT = Rutin hydrate. N = 5 animals.

D

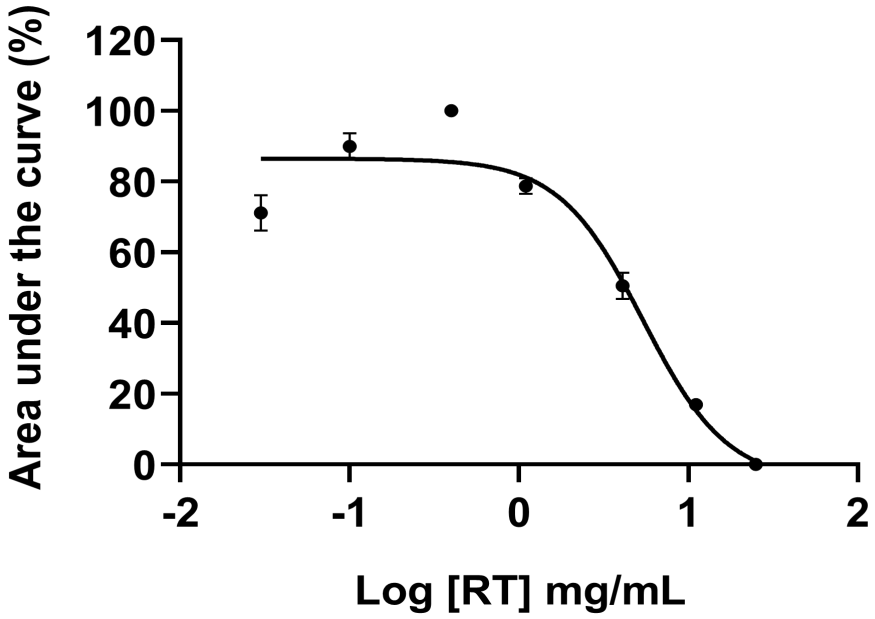


Figure 3.1c A plot of the area under the curve (AUC) showing the inhibitory effect of RT on the cumulative strength and frequency of spontaneous pregnant uterine contractions. RT = Rutin hydrate. n = 5 animals.

A

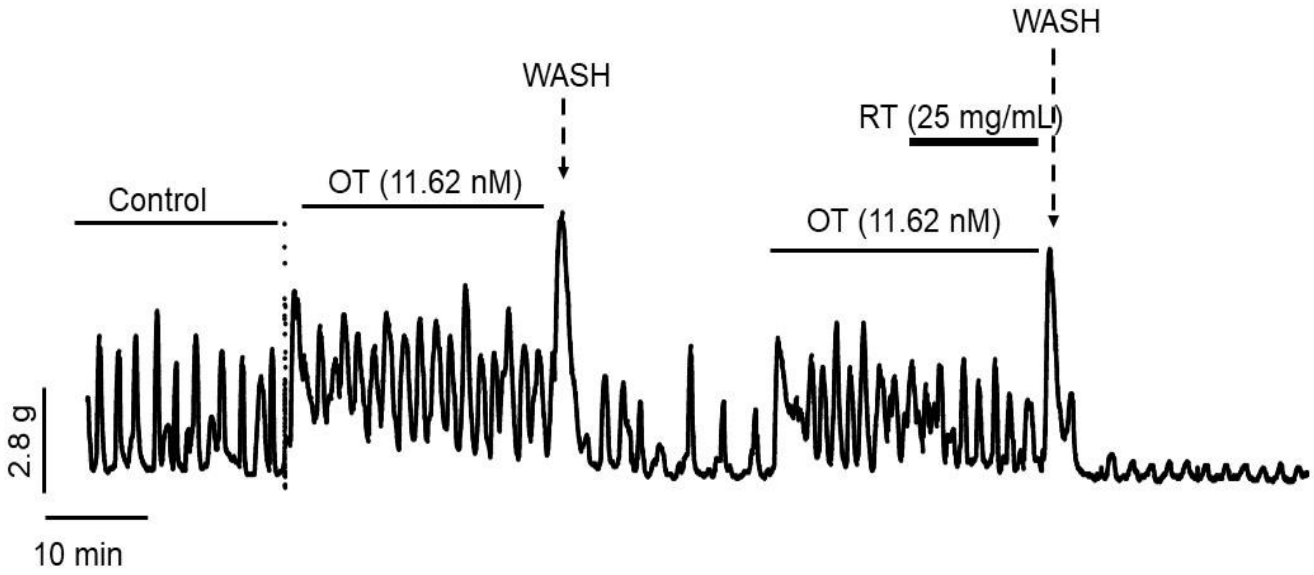
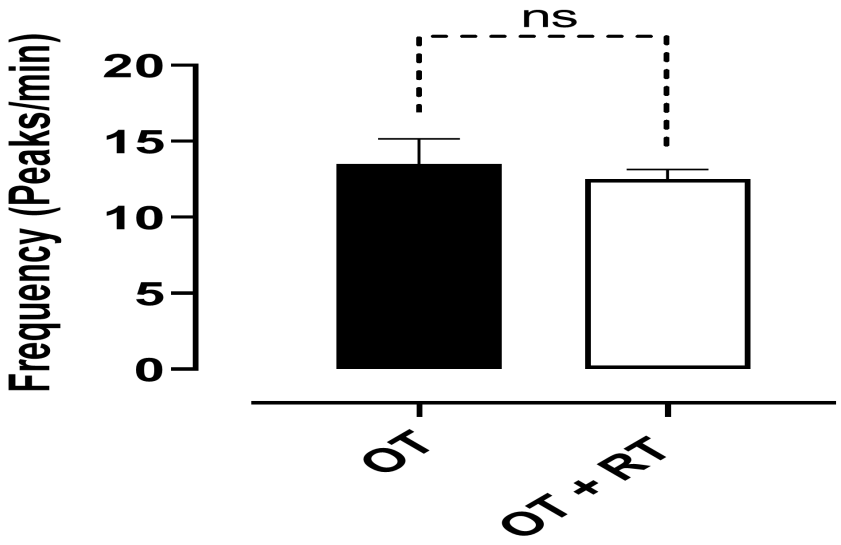
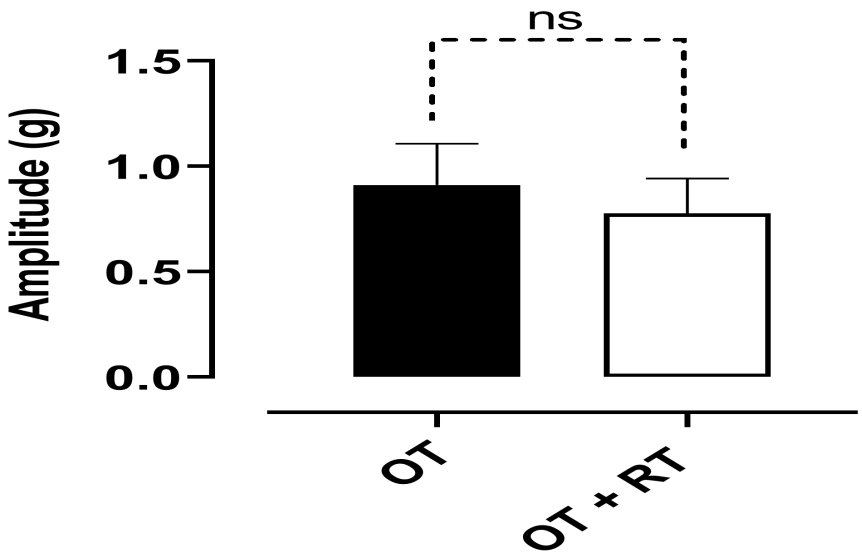


Figure 3.2a Original representative recording showing the effect of RT (25 mg/mL) on oxytocin-induced (11.62 nM) contractions of the pregnant uterus. RT = Rutin hydrate; n = 5 animals; OT = oxytocin.



B



C

Figure 3.2b Bar graph showing the effect of RT (25 mg/mL) on the frequency (B) and amplitude (C) of oxytocin-induced contractions (11.62 nM) in pregnant uterus. RT = Rutin hydrate; n = 5 animals; OT=oxytocin; ns = not significant.

D

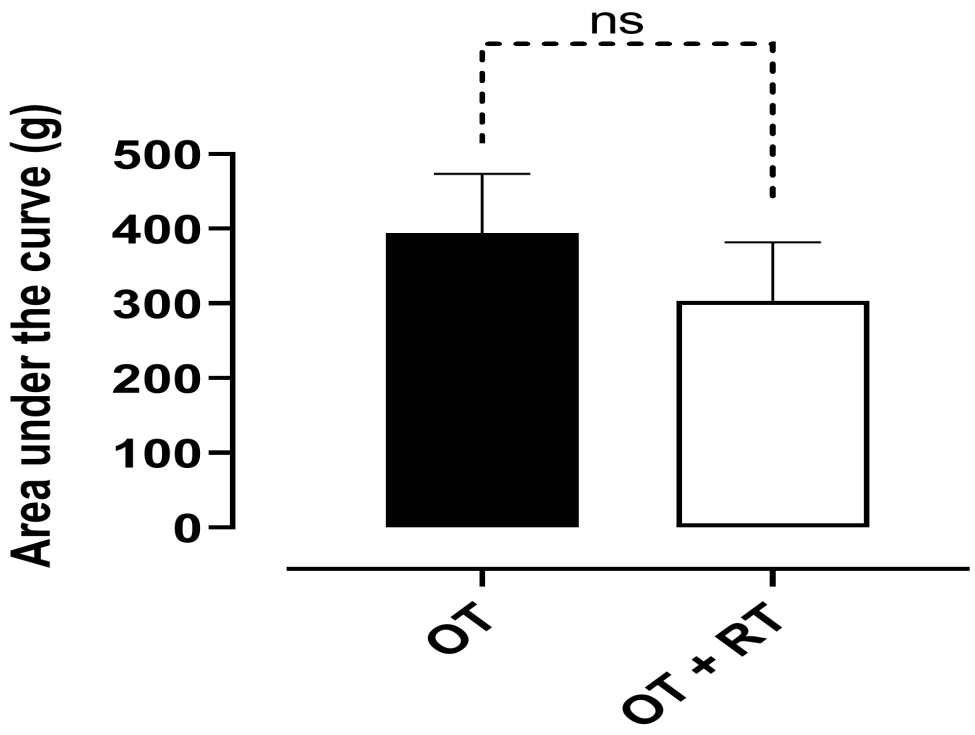


Figure 3.2c Bar chart of the area under the curve showing the effects of RT (25 mg/ml) on the cumulative strength and frequency of oxytocin-induced pregnant uterine contractions (11.62 nM). RT = Rutin hydrate; n = 5 animals; OT = oxytocin, ns = not significant.

A

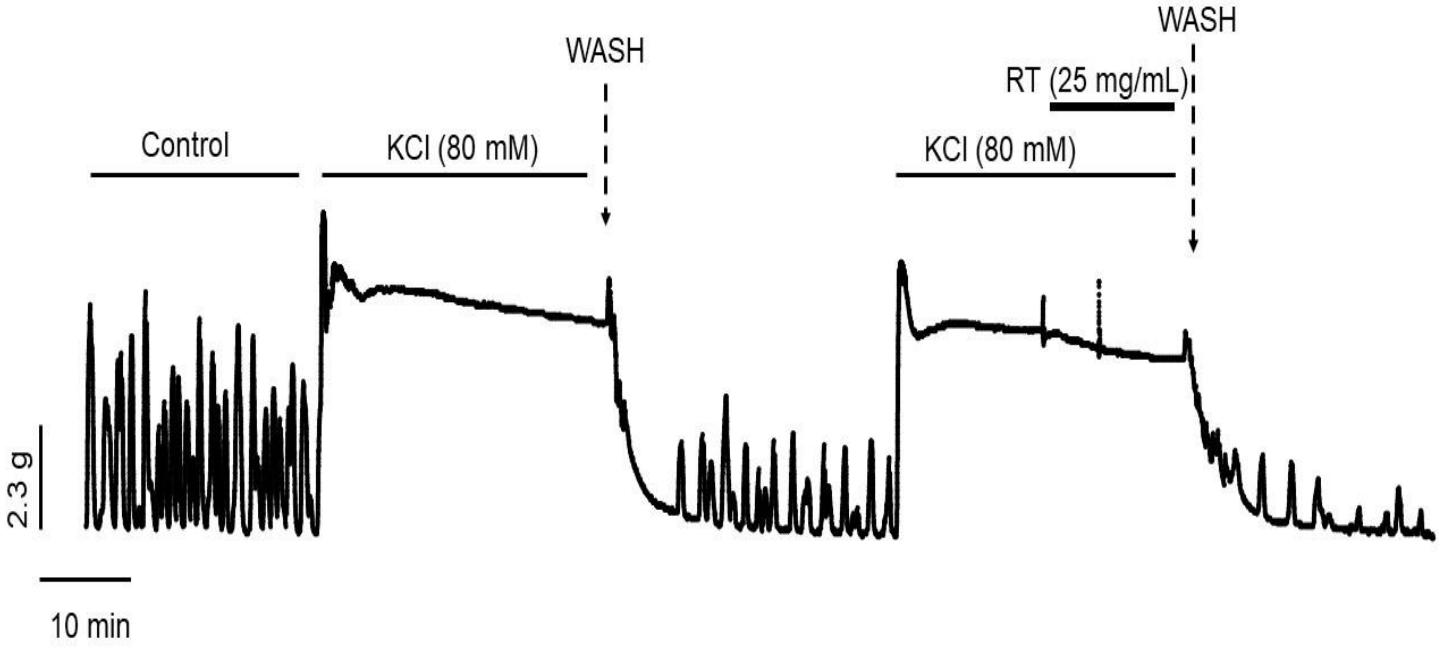


Figure 3.3a Original representative recording showing the effect of RT (25 mg/mL) on KCL-induced contractions (80 mM) of the pregnant uterus. RT = Rutin hydrate; n = 5 animals; KCL = Potassium Chloride.

B

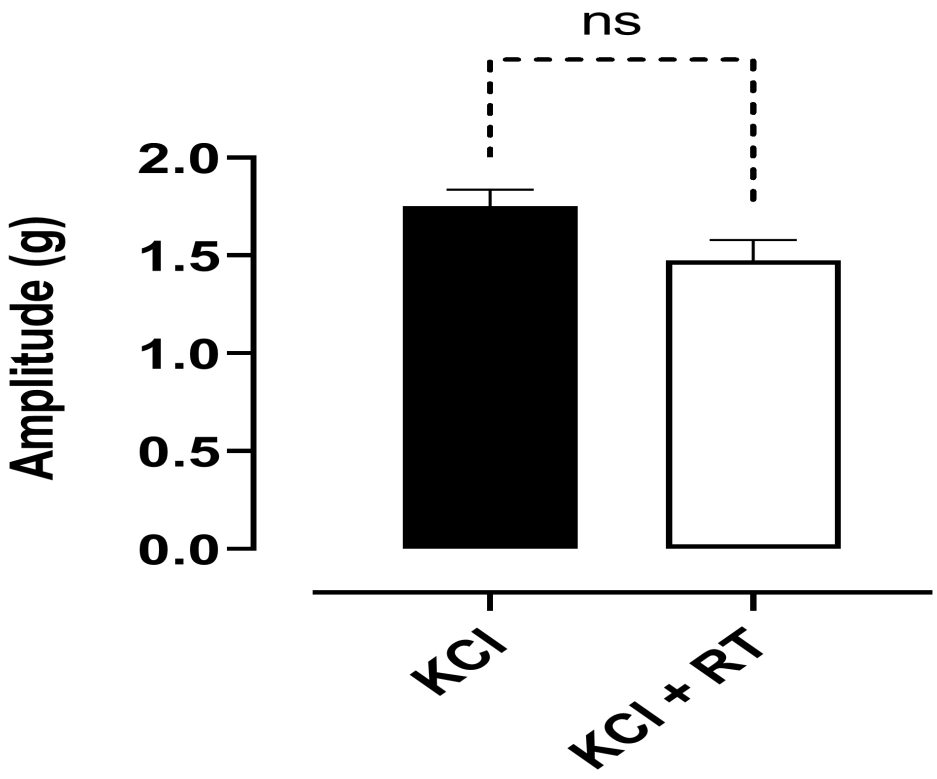


Figure 3.3b Bar graph showing the effect of the RT (25 mg/mL) on the amplitude (B) of KCL-induced contraction (80 mM) in the pregnant uterus. RT = Rutin hydrate; n = 5 animals; KCL = Potassium Chloride; n = not significant.

A

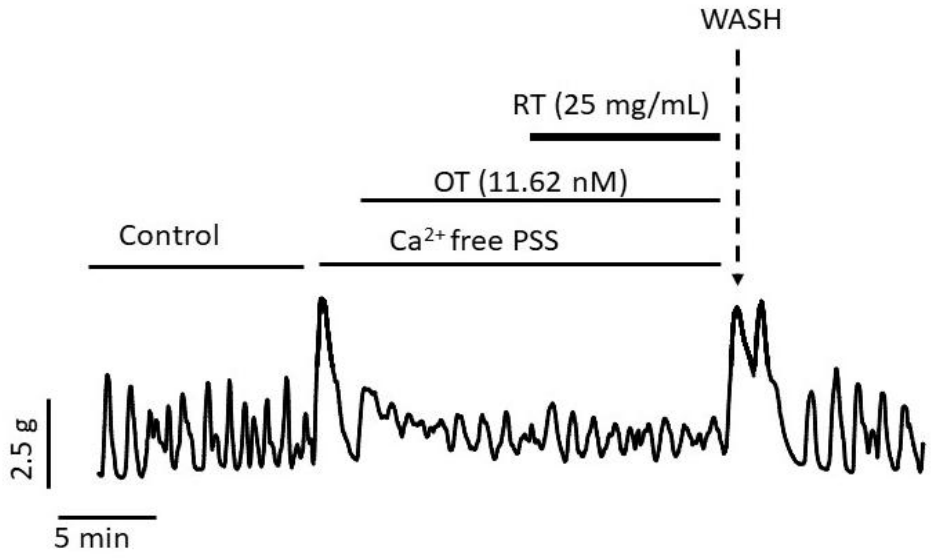
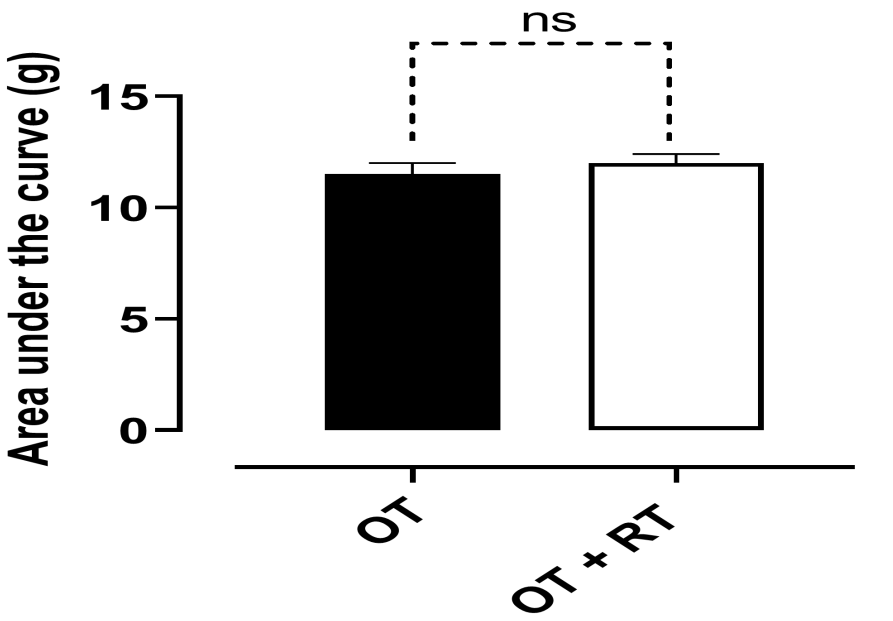
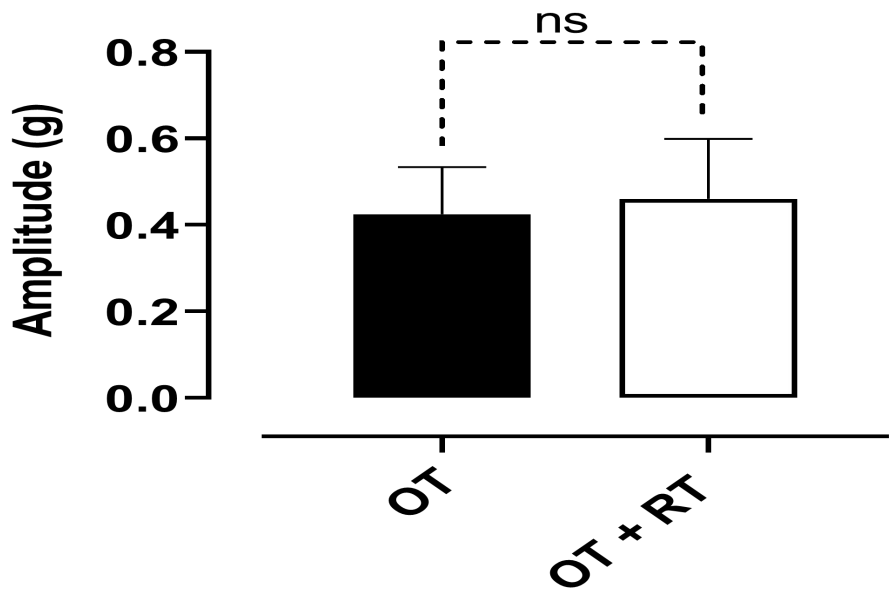


Figure 3.4a Original representative recording showing the effect of RT (25 mg/mL) on oxytocin-induced (11.62 nM) pregnant uterine contractions in a calcium free medium. RT = Rutin hydrate N=5 animals; OT=oxytocin.

B





C

Figure 3.4b Bar graph showing the effect of RT (25mg/mL) on frequency (B) and amplitude (C) of oxytocin-induced pregnant uterine contractions (11.62 nM) in a calcium-free medium. RT = Rutin hydrate; N = 5 animals; OT = Oxytocin; ns = not significant.

D

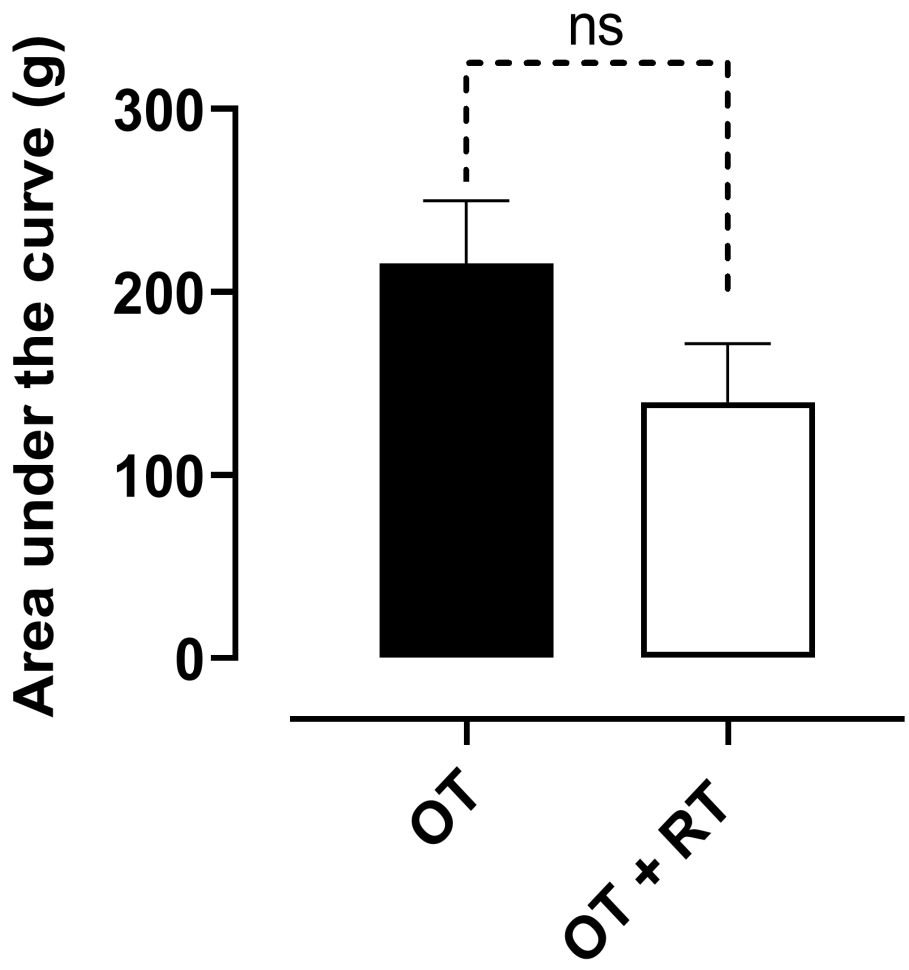


Figure 3.4c A bar chart of the area under the curve (AUC) showing the effect of RT (25mg/ml) on the cumulative strength and frequency of oxytocin-induced pregnant uterine contractions (11.62nM) in a zero-calcium medium. RT = Rutin hydrate; N = 5 animals; OT = Oxytocin; ns = not significant.

CHAPTER FOUR

4.0: DISCUSSION

In this study, our primary research questions focused on investigating the effect of Rutin hydrate (RT), a flavonoid compound, on isolated pregnant mice uterus, determining its dose-dependent effects, and elucidating the mechanisms underlying RT's actions on pregnant uterine contractility. Our objectives were to investigate the effect of RT on the uterine contractions in the pregnant mice, including spontaneous and agonist-induced contractions.

This study's findings revealed that RT inhibited spontaneous uterine contractions in pregnant mouse model via a dose-dependent manner. It however showed no significant effects on agonist-induced contractions produced by oxytocin, high KCL, and oxytocin-induced contractions in a calcium-free medium. Importantly, contractions resumed after washout in all the experiments, suggesting potential reversibility of its effects rather than irreversible damage to the tissue (Loch-Caruso *et al.,* 2003). However, further studies are still needed to investigate the potential toxicity of this compound's use in pregnancy. In order to elucidate the possible mechanism by which RT inhibited spontaneous uterine contractions, it is essential to revisit the uterine cell excitation-contraction coupling mechanism, as this fundamental process seems to underlie the spontaneous contractions observed in the uterus.

Spontaneous uterine contractions are contractions of the myometrium that occur spontaneously and do not require hormonal or nervous input to contract (Garfield & Maner, 2007). These contractions are always present in a thriving uterus, and they are differentiated based on frequency, amplitude, duration and direction of propagation. (Iams *et al*., 2002). Previous studies show that spontaneous uterine contractions play a significant role in the physiology of the

pregnant uterus, but irregularities in these contractions have been implicated in pathologic conditions such as postpartum haemorrhage, preterm delivery, dystocia and uterine atony.

Uterine cell excitation-contraction coupling primarily relies on calcium ions. Spontaneous myocyte contractility involves actin, myosin, and calcium ions. The process consists of several key steps *viz*: Cell membrane depolarization results from extracellular calcium influx through the voltage-gated, L-type calcium channels (VGCC). This depolarization opens the Ryanodine receptors (RyRs) present on the Sarcoplasmic reticulum (SR), releasing intracellular store SR calcium into the cytosol. Increased cytosolic calcium activates calcium-sensitive Myosin Light Chain Kinase (MLCK), which phosphorylates myosin light chain (MLC). Phosphorylated MLC binds to actin, initiating cross-bridge cycling and uterine cell contraction. Cytosolic calcium decreases via plasma membrane calcium ATPase (PMCA) and sarcoplasmic reticulum calcium ATPase (SERCA), transporting calcium ions out of the cell and back into the SR, respectively. Reduced calcium levels deactivate calcium sensitive MLCK, leading to MLC dephosphorylation and decreased affinity for actin, resulting in cell relaxation. Both the release of calcium from the intracellular stores and the influx of calcium from the extracellular space serve as steps to orchestrate spontaneous uterine cell contraction and the reversal causes relaxation via calcium-dependent excitation-contraction coupling (Somlyo, 1985; Pehlivanoglu *et al*., 2013). In this study, RT showed strong inhibition of spontaneous contraction, in a dose-dependent manner, in the isolated pregnant mice uterus. The amplitude and frequency of the spontaneous uterine contractility of the pregnant uterus were significantly decreased. There are several possible mechanisms by which RT could have inhibited spontaneous contractions of the myometrial cells via calcium-dependent excitation-contraction coupling. Some of these mechanisms include: Possible inhibition of calcium ions influx from the extracellular space via blocking of the

voltage-gated calcium channels located on the myometrial membrane. This reduces the amount of calcium ions available for excitation-contraction coupling, leading to inhibition of spontaneous contractions. Possible inhibition of calcium release from the intracellular stores in the SR. This can also reduce the amount of calcium ions available for excitation-contraction coupling, leading to inhibition of spontaneous contractions. Inhibition of MLCK, thus preventing the phosphorylation of MLC, which is necessary for the initiation of the cross-bridge cycling and the contraction of the myometrial cell (Pehlivanoglu *et al*., 2013).

Following the observation of the relaxant effect of RT on spontaneous uterine contractions, suggesting its possible tocolytic effect, it became necessary that we extended our investigations to agonist-induced contractions, as further steps to determine the potential mechanism of action underlying RT's relaxant effects on the pregnant uterus.

Oxytocin-induced uterine contractions generally serve as a model for testing the efficacy of potential tocolytic agents (Arrowsmith *et al*., 2018). Testing if RT which inhibits spontaneous myometrial contractions also inhibits oxytocin-induced contractions is important because oxytocin is one of the primary hormones involved in uterine contractions and will indicate whether RT's inhibitory effect on the uterine contraction is associated with the extracellular and/or intracellular Ca^{2+} influx (Pierzynski *et al.,* 2004, Uchendu & Bafor, 2023). Recall that oxytocin, both endogenous and exogenous, induces uterine contractions in the myometrial cells via several mechanisms including: Activation of oxytocin receptors: Oxytocin binding to myometrial cell oxytocin receptors (OTR), triggers activation of G-protein coupled receptors. This stimulates phospholipase C (PLC) responsible for secondary messenger generation (diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) generation) by the hydrolysis of phosphatidylinositol bisphosphate (PIP2). IP3 then induces calcium release from the SR,

elevating intracellular calcium levels, which in turn, activates MLCK to phosphorylate MLC, initiating cross-bridge cycling and cell contraction. This stimulation of calcium entry is regarded as the most prominent effect of oxytocin (Osilla & Sharma, 2023). Possible contribution from extracellular calcium also occurs with oxytocin and occurs via activation of voltage-gated, L-type calcium channels (Wray *et al*., 2001). Inhibition of calcium efflux mechanisms: Oxytocin decreases calcium efflux by inhibiting calcium ATPase of the myometrial cell membrane, which pumps calcium from the intracellular to the extracellular space (Arrowsmith *et al*., 2010). Production of prostaglandins: Oxytocin-induced contractions initiate prostaglandin production, thus intensifying uterine contractions. The process involves oxytocin binding to myometrial OTRs, hence activating G-protein coupled receptors. Activation of G-protein coupled receptors stimulates phospholipase A2 (PLA), cleaving arachidonic acid from the cell membrane. Arachidonic acid is converted to prostaglandins by cyclooxygenase (COX) enzymes. Prostaglandin release from myometrial cells further amplifies uterine contractions (Vrachnis *et al*., 2011). Moreover, oxytocin binding to myometrial OTRs also activates another G-protein coupled receptors. This receptor activation triggers Mitogen-activated protein kinase (MAPK) and the Rho kinase pathways. OTR and MAPK both result in increased PLA activity, necessary for prostaglandin production, thereby contributing to the contractile effect. While OTR and RhoA-ROCK cascade leads to increased MLC phosphorylation, further enhancing myometrial contraction in response to oxytocin (Vrachnis *et al*., 2011). Endogenous oxytocin also stimulates positive feedback of more oxytocin release by oxytocin release from self or nearby cells via autocrine and paracrine mechanisms, respectively (Higashida, 2016). Studies have shown that all these mechanisms are responsible for the higher amplitude and frequency observed in oxytocin-induced uterine contractions when compared to the spontaneous contractions serving as control

in Figure 3.2a. In this study, RT's Emax dose (25 mg/mL) showed no significant inhibitory effect on both the amplitude and frequency of oxytocin-induced contractions (11.62 nM). This suggests that RT does not inhibit oxytocin-induced uterine contractions. There may be several possible explanations for this occurrence, some of which we have attempted to provide. The inhibition of spontaneous uterine contractions but not OT-induced contraction by RT may suggest that it's relaxant effect may not be associated with the extracellular and/or intracellular Ca^{2+} influx triggered by oxytocin. OT, in addition to its conventional Ca^{2+}pathway, also facilitates myometrial contractions via mechanisms that are distinct from calcium-dependent ones, such as the MAPK/RhoA-ROCK cascade, this further increases the intensity and amplitude of its induced contractions in the uterus compared to spontaneous contractions. This heightened effect might overwhelm the single dose relaxing effects of RT (25 mg/ml) on its induced contractions. RT may even have a different mechanism of action that selectively inhibits spontaneous uterine contractions but not oxytocin-induced contractions. For instance, via phosphodiesterase-4 inhibition or β-Adrenoceptor agonism.

At this stage, it was still unclear if RT interacts or not with either/both the intracellular and extracellular sources of calcium into the cytosol. The VGCCs play a role in both spontaneous and oxytocin-induced contractions. They drive extracellular Ca^{2+} entry into the cytosol of myocytes. In order to investigate the likely interaction of RT with VGCCs, RT was tested on high KCL-induced uterine contractions. High KCL induces contraction via a straightforward mechanism. This process involves depolarization of the myocyte membrane, activation of the voltage-operated, L-type calcium channels (VOCC) located on the myometrial membrane, resulting in elevated cytosolic free calcium levels following influx of calcium from the extracellular fluid, activation of the calcium-calmodulin-dependent MLCK, MLC

phosphorylation and subsequent contraction. (Ratz *et al*., 2005). RT did not substantially alter high KCL-induced uterine contractions (Fig 3.3a). The amplitude of contractions induced by KCL (80 mM) remained largely unchanged, as illustrated in Fig 3.3b. This may suggest that RT induced uterine relaxant effect is likely not related to the inhibition of extracellular Ca^{2+} influx.

As earlier mentioned, intracellular calcium present in SR stores contributes to uterine contractility. Hence, the activity of RT was further investigated on OT-induced contractions in a calcium-free PSS medium. This helped demonstrate the effect of RT on the mobilization of calcium from the myometrial intracellular store for the regulation of myometrial smooth muscle contractions. OT can elicit uterine smooth muscle contraction even in calcium-free physiological salt solution, suggesting that oxytocin-induced contractions can occur via an extracellular calcium-independent mechanism. (Tahara *et al*., 2002). RT's inhibitory dose (25 mg/mL) showed no significant effect on oxytocin-induced contractions (11.62 nM) in the absence of calcium containing PSS. This suggests that RT seems not to have possible interaction with the mobilization of Ca^{2+} from its intracellular stores.

Having examined the outcomes of this experimental findings, it is essential to now turn our attention to the evaluation of these results in the light of our initial hypotheses. We hypothesized that RT would exert a positive relaxation effect on spontaneous and agonist-induced uterine contractions, with a presumed link to the modulation of calcium activity inhibiting these contractions, in a dose-dependent manner. Contrary to our expectations, our findings revealed that although RT exhibited a positive relaxation effect on spontaneous uterine contractions, agonist-induced contractions remained largely unaffected. Our findings also suggest a divergence from the calcium-mediated pathway we initially proposed. While we observed a dose-dependent relaxant effect on spontaneous pregnant mouse uterine contractions, the same

Emax concentration (25 mg/mL) did not yield significant effects on agonist-induced contractions. Interestingly, resumed contractions post washout of the compound may suggest that its relaxant effect might be reversible. Nonetheless, it is essential to highlight again the need for additional toxicity studies to comprehensively address this aspect.

Despite the scarcity of studies analysing the effects of this compound, particularly on pregnant uterine smooth muscle contractility, it is valuable to draw comparisons with the available body of existing literature in this field. From previous studies, RT resulted in a concentration-dependent relaxation of aortic rings precontracted with phenylephrine and caused endothelium-dependent relaxation of the isolated rat aorta probably via nitric oxide-guanylyl cyclase mechanisms (Zhou *et al*., 2006; Michalikova *et al*., 2019). While our study focuses on uterine smooth muscle contractility instead, our findings also reveal a relaxing effect, albeit on only pregnant mice spontaneous uterine contractions, where calcium modulation appears to play a significant role. This suggests the necessity for further investigation of RT's effect on selective α-1 adrenergic receptors and the guanylate cyclase-cyclic GMP-PKG pathways as potential mechanisms of action to cause relaxation on the pregnant uterine smooth muscle contractility.

In another study, RT was also shown to produce relaxation in rat ileum and trachea tissue precontracted by Ach response, showing a likely inhibition due to possible anticholinergic activity (Sekha & Al-Habib, 2013). Interestingly, in our study, RT relaxed pregnant spontaneous uterine contraction via a possible calcium-independent pathway. Hence, this comparison also raises the possibility that RT's effect could be linked to its potential sympathetic/parasympathetic effects on the uterus. Further research is recommended to explore this potential mechanism of action on uterine tissues.

Sekha & Al-Habib (2013) study showed non-significant relaxation in KCL-precontracted trachea, the study implied that RT had only minimal calcium antagonistic activity in the tracheal tissues. Our findings align with this observation, as RT showed no significant inhibitory effect on KCL-induced pregnant uterine contractions, a method typically used to assess its impact on extracellular calcium mobilization. Comparing these results, it may seem that RT exhibits minimal calcium antagonist activity, not only in the trachea, but also in the uterus, where calcium plays a pivotal role in contractions. This further emphasizes the need for further investigation into alternative mechanisms of action for RT, particularly for use as a tocolytic agent in reproductive health.

CHAPTER FIVE

5.1: CONCLUSION

This study presents the scientific evidence that Rutin hydrate exerts relaxing effects on spontaneous uterine contractions but does not significantly affect agonist-induced contractions in isolated pregnant mice uterine tissues. This phenomenon maybe attributed to the fact that RT does not interfere with calcium-dependent mechanisms of action in the uterus, including the blocking of calcium influx from the extracellular space through the voltage gated, L-type calcium channels, or inhibiting calcium release from the intracellular stores. This study also represents, to the best of our knowledge, one of the initial investigations into the effect of RT on isolated pregnant mice uterine contractions, both spontaneous and agonist induced. It therefore suggests that RT requires further investigation as a potentially new drug treatment or as a complement to already existing approaches, via pathways other than calcium antagonism, for managing conditions requiring uterine contractility inhibition during pregnancy, such as in preterm labour.

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