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THE INFLUENCE OF ALPHA-TOCOPHEROL ON THE SMOOTH MUSCLE CONTRACTIONS: THE SIGNIFICANCE OF CYCLOOXYGENASE FUNCTION

Ph.D. Thesis

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List of abbreviations

CAT	catalase
COX	cyclooxygenase enzyme
COXi	cyclooxygenase enzyme inhibitor
CSM	cervical smooth muscle
DCs	dentric cells
GI	gastrointestinal
GSH-Px	glutathione peroxidase
IU	international unit
LOX	lipoxygenase enzyme
NASH	nonalcoholic fatty liver disease
NSAID	non-steroidal anti-inflammatory drug
OSI	oxidative stress index
PG	prostaglandin
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PKCα	protein kinase C alpha
PLA2	phospholipases A2
RDA	recommended dietary allowance
ROS	reactive oxygen species

SOD	superoxide-dismutase
TXA2	tromboxanA2
WHO	World Health Organization

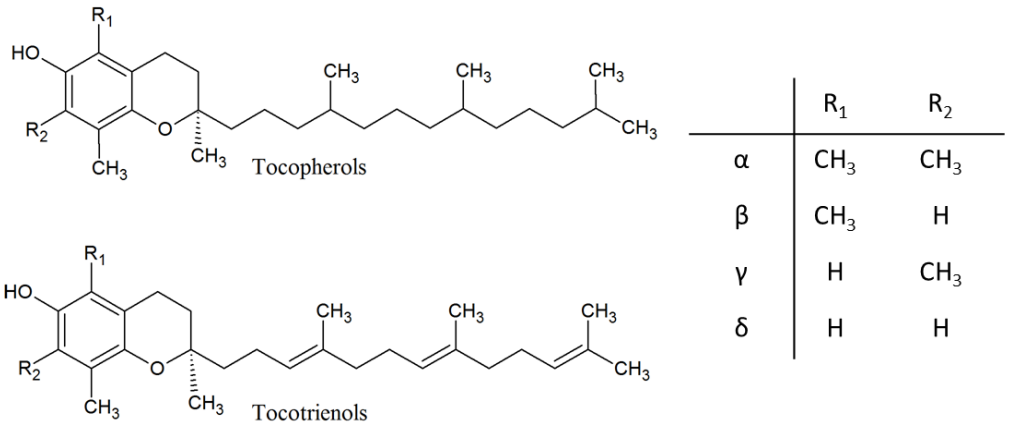
Contents

1. Introduction

1.1. Overview of Vitamin E

In 1922, Evans and Bishops published the first article about the discovery of vitamin E.[1] Then, in the next decades the field of biological activity of vitamin E has been widely investigated. In the first few years, vitamin E were regarded as only an antioxidant compound, but nowadays it became clear that its mechanism of action is extremely complex.

Vitamin E is crucial for the normal physiological functions. It is classified to the group of fat-soluble vitamins and like most of them, it cannot be synthesized in the human body, thus it is necessary to get into from other sources e.g. food or dietary supplements. The recommended intake dose for adults is 15 mg/22.4 IU/day by RDA.[2] In the nature, vitamin E exists as a mixture of eight forms such as α-, β-, γ-, δ-tocopherols, and tocotrienols. Each analogue consists of a chromanol ring and a 16-carbon phytyl-like side chain, wherein tocopherols are saturated while tocotrienols have three double bonds.[3] The difference between isoforms of tocopherols and tocotrienols is the number and position of the -CH_3 group of the chromanol ring. All forms possess peroxyl radical scavenger ability due to the –OH group of chromanol ring.[4] **(Figure 1.)**



	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 1. *Chemical structure of tocopherols and tocotrienols.*[5]

Numerous *in vitro* and *in vivo* studies have demonstrated that the activity of the analogues is quite various. α-tocopherol is the most effective against vitamin E deficiency disease and it can reach the highest plasma concentration. Based on these facts, α-tocopherol is the only which can be named as vitamin E.[6]

The low level (< 5 mcg/ml in adults)[7] of Vitamin E in blood can lead to muscle weakness, areflexia, Purkinje-cells degeneration, ataxia, mostly in ages 5 and 15 years[8;9] and it may increase the risk of several diseases, including atherosclerosis, inflammation, non-alcoholic fatty liver diseases NASH, cancer and neurodegenerative diseases.[10–14]

The mechanism of action of vitamin E has not yet been fully clarified. On the one hand, as an antioxidant, α-tocopherol can protect against oxidative damage in membranes and lipoproteins by way of scavenging of reactive oxygen and nitrogen species and inhibiting of lipid peroxidation.[15] In organism, free radicals are liberated during metabolic process. In a physiological case, there is a balance between free radicals and antioxidants wherein the parts of the protective system are CAT, GSH-Px, SOD, vitamin C, carotinoids and vitamin E. This balance can be shifted by some environmental effects, hereby certain tissues and proteins such as DNA, neurons, erythrocytes and retina which are sensitive specifically to the amount of free radicals can be damage. During the antioxidant process, α-tocopherol is oxidized to α-tocopheroxyl, then it can transform back to α-tocopherol by ascorbate.[16] **(Figure 2.)** Moreover, beyond of the protection, tocopherol participates in the action of redox regulated enzymes and transcriptions factors i.e. protein kinase C, protein phosphatase 2A, protein tyrosine phosphatase 1B, NFκB and others.[17]

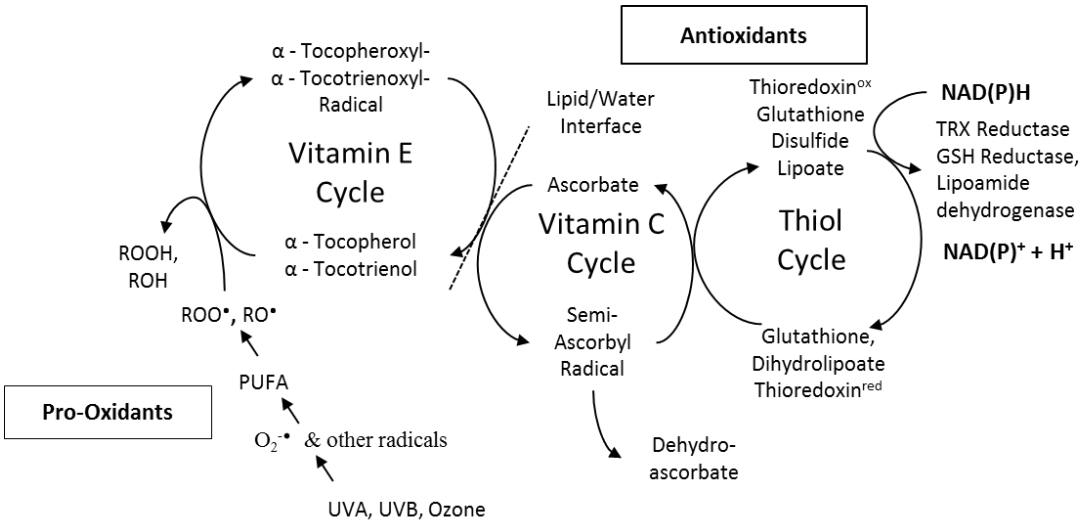


Figure 2. *The mechanism of antioxidant system in human body.*[16]

On the other hand, α-tocopherol also has a non-antioxidant molecular mechanism of action. Many studies report that α-tocopherol binds non-covalently to several proteins and affects their efficacy. It has already been shown that α-tocopherol is able to bind transport proteins immediately such as α-tocopherol transfer protein, CD36/FAT scavenger receptor/fatty acids transporter, tocopherol associated proteins 1, 2 and 3 and enzymes: PLA2, PKCα, 5-,12-, 15-lipoxygenase (5-, 12-, 15-LOX), cyclooxygenase 1 and 2 (COX-1, COX-2), which play an important role in signal transduction and production of active lipid mediators.[18] Moreover, tocopherol alters the interaction of membrane-proteins and the protein translocation to plasma membrane.[19] Additional non-antioxidant property of vitamin E is that it may regulate gene expression.[20]

Role of vitamin E in reproduction

Vitamin E was first described as a necessary dietary factor for reproduction in human and animals.[1,21] In fact, vitamin E plays a significant role in pregnancy, it protects foetus, placenta and uterus from oxidative damage, in addition, it improves microcirculation of placenta and myometrium and increases the level of progesterone.[22] Normally, the plasma concentration of vitamin E is enhanced during pregnancy,[23] furthermore the oxidative stress index (OSI) of pregnant uteri remains low while its antioxidant status can be high in rats.[24] Based on these facts, normally it is not necessary to supplement extra vitamin E for pregnant women. At the same time, if their dietary is not adequate or in case of inflammatory bowel diseases or cystic fibrosis, vitamin E should be supplemented. Since, vitamin E deficiency in pregnant woman can be associated with vascular endothelial injury, preeclampsia, low-birthweight placental abruption, abortion and premature birth, the background of these pregnancy outcomes may be the increased oxidative stress and/or the impaired antioxidant mechanism.[25] In non-pregnant woman, the enhanced serum level of α-tocopherol may raise the risk of anovulation.[26] Moreover, it was shown that vitamin E administration before and during menstruation decreased the dysmenorrhea-caused pain and menstrual bleeding.[27]

Role of vitamin E in respiratory system

World-widely, the incidence of asthma is fairly high; according to WHO evaluation, 339 million people suffer from it.[28] This is the most common chronic disease among children. Asthma is a heterogeneous respiratory disease which is characterised by chronic airway inflammation, reversible airflow obstruction and enhanced bronchial reactivity. Its main symptoms are wheezing, breathlessness, chest tightness, cough and sputum production. In most of the cases, asthma starts from childhood when the allergic sensitization is coming off. The allergens bind to IgE on dentric cells (DCs) and this process induces the production and release of inflammatory mediators such as allergic-specific T helper type cells Th2, Th17, Th9, interleukin (IL)-17A, IL-17F, IL-22. [29] Since, asthma is correlated with inflammation, and vitamin E possesses an anti-inflammatory ability, in the last decade it has been suggested that vitamin E may help the treatment or prevention of asthma. Both *in vitro* and *in vivo* studies demonstrated that vitamin E isoforms can reduce the intensity of asthma response, furthermore it diminishes the airway hyper-reactivity, production of mucus and liberation of inflammatory cytokines. In addition, it can inhibit the eosinophil and neutrophils infiltration to lung and IL-4 expression in T-cells. It seems that low level of vitamin E in maternal blood can affect the development of childhood asthma[30,31] and may influence the growth of foetal lungs.[32] Finally, vitamin E can participate against infection of respiratory system such as Streptococcus pneumonia[33] and secondary Staphylococcus aureus pneumonia in mice.[34]

1.2. The cyclooxygenase (COX) pathway

The COX pathway is a part of the arachidonic acid cascade, in which the phospholipids of cell membrane transform to different types prostanoids such as thromboxane, prostacyclin and prostaglandin. Several enzymes catalyse this conversion, however COX is amongst the most important. COX or in other name PGH synthase, possesses double activities: it catalyses arachidonic acid to PGG2, while by peroxidase activity, it transforms PGG_2 to PGH_2. There are two isoforms of COXs, COX-1 and COX-2. COX-1, as a constitutive form, is responsible for the synthesis of PGs which help the maintenance of the physiological functions, while COX-2 is induced by inflammation.[35] At the same time, it is also generally accepted that in physiological condition, COX-2 is presented only in low amounts. Furthermore, in tissues the expression and activity of COXs can be different.[36]

In the past years, it has been established that due to the peroxidase activity of COXs, reactive oxygen species (ROS) can be released and on the other hand, ROS can modulate the COX pathway. ROS and oxidative stress induced the expression and up-regulation of COXs, but mainly COX-2.[37]

1.3. COX inhibitors

COX inhibitors are grouped by selectivity of inhibition. Thus, selective COX-1 inhibitors i.e. SC-560, selective COX-2 inhibitors i.e. rofecoxib and non-selective COX inhibitors i.e. diclofenac can be marked. Since, there is no inhibitor with 100 % selectivity, their selectivity can be typified as log IC_{50} COX-1/COX-2 ratio. The inhibition effect may be irreversible or reversible, the best-known irreversible COX inhibitor is acetylsalicylic acid. COX inhibitors diminish the production and release of prostaglandins, prostacyclin and thromboxane. Many of the COX inhibitors are applied as non-steroidal anti-inflammatory drugs (NSAIDs). The NSAIDs are one of the most frequently used medicines in the world, in most of the cases, they are available without prescription. The main indications of these compounds are pain, inflammation and fever. Despite of these, low dose about 100 mg per day of aspirin is used for thrombocytes aggregation inhibition, indomethacin for acute gout and close a ductus arteriosus in neonates. NSAIDs also have side effects, such as ulcers, GI bleeding, bronchoconstriction and allergic reaction.[38,39]

1.4. Role of prostaglandins in smooth muscle contraction and cervical ripening

It is well known that prostanoids (PGI2, PGE2, PGD2 and PGF2α, thromboxane A_2) play an important role in contraction or relaxation of smooth muscles. These effects are evolved via G protein-coupled prostaglandin receptors. Prostaglandin receptor subfamily are typified by letter code such as PGs e.g. PGE_2: EP1-4, PGF2α: FP, PGI_2: IP, PGD_2: DP, TXA_2-TP. Generally, the expression of prostanoid receptors is tissue specific and their provoked cell signalling is very complex due to isoform of subtypes **(Table 1.)**.[40,41]

Type	Subtype	Isoform	G Protein	Second Messenger
DP			G_s	cAMP ↑
EP	EP_1		Unidentified	Ca^{2+} ↑
	EP_2		G_s	cAMP ↑
	EP_4		G_s	cAMP ↑
	EP_3	EP_{3A}	G_i	cAMP ↓
		EP_{3B}	G_s	cAMP ↑
		EP_{3C}	G_s	cAMP ↑
		EP_{3D}	$G_{i,}$ $G_{s,}$ G_q	cAMP ↑, cAMP ↓ PI response
FP			G_q	PI response
IP			G_s, G_q	cAMP ↑, PI response
TP		TPα	$G_{q,}$ G_i	PI response, cAMP ↓
		TPβ	$G_{q,}$ G_s	PI response, cAMP ↑

Table 1. *The summarized table about prostanoids receptors***.**[41]

In myometrium, mainly the PGE_2 is responsible for contraction and relaxation. The EP1 and EP3 receptors induce contractions while EP2 and 4 mediate relaxations. Compared to non-pregnant and pregnant uteri the level of COXs, PGE_2, $PGF_{2\alpha}$, and their receptors are varied, in addition during pregnancy, their amount is also changed. Since, parturition is an extremely complex process, some question has still remained open about the exact mechanism.[42]

Prostanoids are also associated with airway contraction and relaxation. They are synthesized in the mast cell, epithelium and smooth muscle of the airway. In fact, PGE_2 and PGI_2 can cause bronchodilation via DP_1, EP_2/EP_4 and IP_1 receptors, while PGD_2, $PGF_{2\alpha}$ and TXA_2 can evoke bronchoconstriction by nonselective thromboxane and EP1/EP3 and FP receptors.[43,44] In respiratory diseases which involve bronchoconstriction like asthma or COPD, imbalance is evolved in production of prostanoids by inflammation.[45]

The cervical ripening is crucial part of parturition. During pregnancy, the cervix undergoes a drastic transformation which is induced by various endogenous substances such as PGs, cytokines and hormones. At the end of pregnancy, both levels of COX-1 and COX-2 enzymes are also increased. Moreover, it is possible that the liberation of COX-2 related PGs liberation has higher importance in cervical ripening and spontaneous labor and out of prostanoids, PGE_2 and $PGF_{2\alpha}$ play a more potent role in this process.[42,46] In

clinical practice, the synthetic PGE1 analogue misoprostol and PGE_2 formulation dinoprostone are used for the induction of labour.[47]

1.5. Interaction between tocopherols and cyclooxygenase enzymes

Only a few studies were published about the association of vitamin E and COXs. Wu et al.[48] reported that vitamin E inhibited the activity of COXs in human aortic endothelial cells. Moreover, vitamin E can affect different steps of the arachidonic acid cascade, but this effect may be diverse in tissues. According to literature, prostaglandin E2 production was reduced in mouse[49,50] and rat[51] macrophages by vitamin E. Analogues of tocopherols have different effects on COX. Alpha-tocopherol succinate inhibited more efficiently the LPS-stimulated PGE2 production in macrophages and also the COX activity in human lung epithelial cells than other analogues.[52,53]

2. Aims

The mechanism of action of vitamin E is still not fully understood. In addition, based on literature data we hypothesized that it may be able to alter the COXs activity. Thus, the focus of my PhD thesis was to determine how α-tocopherol succinate modifies the effects of COX-inhibitors on several types of smooth muscles and cervical ripening. Accordingly, the following aims were set:

1. Since, prostaglandins liberated by COX enzymes have a crucial role in development of uteri and tracheal smooth muscle contraction, the first purpose of our study was to investigate the effects of non-selective COX inhibitor diclofenac, selective COX-2 inhibitor rofecoxib and selective COX-1 inhibitor SC-560 alone and after pre-treatment of tocopherol in non-pregnant, 22-day-pregnant rat uteri and trachea tissues *in vitro.*

2. Prostaglandins also participate in cervical ripening process. Hence, the second aim of the study was to examine the action of tocopherol and mentioned COX inhibitors on non-pregnant and 22-day-pregnant rat cervical resistance *in vitro*.

3. The third aim of the study was to analyse how tocopherol alters the activity of COX-1 and 2 in uteri, cervical and tracheal tissues.

4. As we hypothesized that tocopherol may modify the effects of COX inhibitors via modification of COX enzymes activity, the fourth aim was to observe how tocopherol and COX inhibitors applied alone and in combination change the time of the initiation of parturition in rats *in vivo*.

3. Materials and methods

3.1. Housing and mating of the animals

All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission number: IV/198/2013). The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII).

The applied Sprague-Dawley rats were stemmed from INNOVO Ltd. (Gödöllő, Hungary). For the animals, controlled temperature (22 ± 3 °C), 30-70 % relative humidity, 12 h dark-light cycle was ensured, additionally they were fed standard rodent pellet (Charles-River Laboratories, Budapest, Hungary) and were given tap water *ad libitum*.

To the mating of rats, the females (180-200 g) were chosen by their oestrous phase, which was measured by an Oestrus Cycle Monitor EC40 (Fine Science Tools, Foster City, CA, USA). The mature males (240-260 g) and females in oestrus were placed separately into the mating cage. It was divided with a time-controlled movable metal door which was opened before dawn. In the morning, vaginal smear samples were taken from female animals and under a microscope at a magnification of 1200x, presence of sperm was searching. If there was copulation plug or the vaginal smears were positive the copulation was successful. In these cases, females were separated and the initiation of pregnancy were calculated from this day.

3.2. *In vitro* studies

22-day-pregnant and non-pregnant rats in oestrous phase were used for *in vitro* studies.

3.2.1. Organ bath studies

Before preparation of uteri, trachea and cervix, animals were terminated by CO_2 inhalation. Moreover, in any cases the organ bath was heated at 37 °C and carbogen (95% O2 + 5% CO2) was bubbled into the chambers. The tension of cervix, the contraction of uterus and the tone of trachea were measured with gauge transducer (SEN-03; MDE Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition

System (MDE Ltd., Budapest, Hungary). α-tocopherol-acid-succinate (Sigma-Aldrich Hungary Ltd.) which was administered for organ bath studies, was dissolved in mixture of ethanol 96% : Macrogol 400 (1:14) and diluted further in Macrogol 400. Tissues were incubated for 1 h with a buffer renewal in every 15 minutes. The samples were equilibrated for another 60 min with tocopherol (10^{-7} M); it was added to tissues after every wash of buffer solution. The control preparations were incubated for 1 h without tocopherol

3.2.1.1. Preparation of uteri

After the termination, the abdominal wall was excised and the two horns of uterus were pried out. Ovarium and cervix were disconnected and the two horns of uterus were cut into 5-mm-long muscle rings. In case of pregnant rats, after foetuses and placentas removal, 5-mm-long samples including the sites of implantations were sliced. The tissues were mounted vertically in an organ baths containing 10 ml de Jong buffer (composition in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose, pH 7.4). The initial tension of uteri samples was set to 1.5 g. After the incubation period, the control contractions of uteri were evoked with 25mM KCl and the cumulative dose-response curves of non-selective COXi diclofenac (10^{-9}–10^{-5} M) and COX-2 selective inhibitor rofecoxib (10^{-10}–10^{-5} M) were obtained.

3.2.1.2. Preparation of cervix

The cervices were separated from the two horns of 22-day-pregnant and non-pregnant uterus, then their two rings were cut with razorblade. The samples were mounted vertically by hooks in an organ bath containing de Jong solution (composition in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose, pH 7.4). Initial tension was set to 1.00 g. The cervices were stretched in growing steps and were allowed to relax for 5 min, the tension after 5 min was read from the record by the analysing software. After every 5 min, the next initial tension was set, in 1-g steps between 1 and 12 g. The tension was set up manually via the fine control screw of a gauge transducer. The developed stress-strain curves had a sawtooth shape. In the evaluation of cervical resistance, the initial tension of the cervix was plotted versus the stretch after 5 min. Straight lines were suited by linear regression and the slopes of the lines were applied to

express the degree of resistance. A steeper slope reflected a higher resistance.[54] **(Figure 3)**

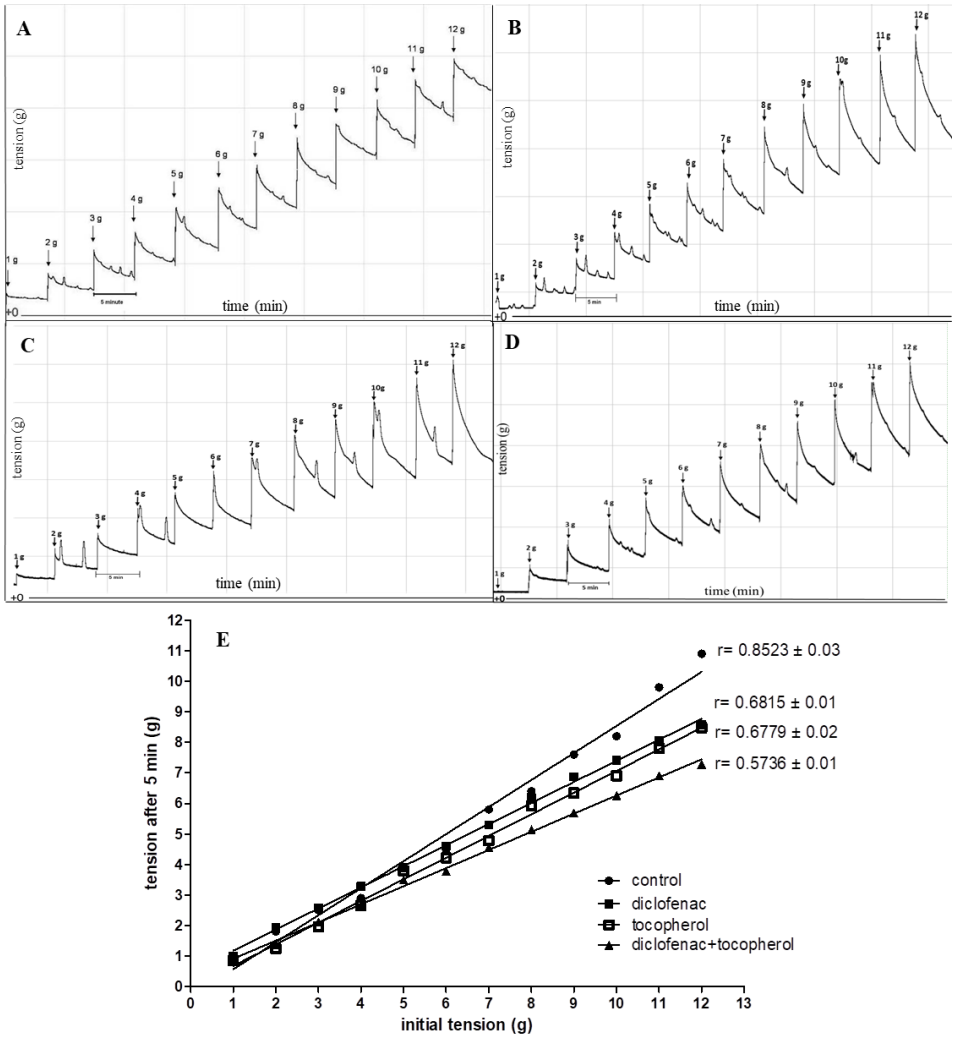


Figure 3. *The recorded saw-tooth shape stress-strain curves of 22-day-pregnant cervices.* ***(A)*** *control;* ***(B)*** *in the presence of diclofenac (10^{-6} M);* ***(C)*** *after pretreatment with α-tocopherol (10^{-7} M) and diclofenac (10^{-6} M);* ***(D)*** *α-tocopherol alone (10^{-7}M). The linear regression of curves A; B; C; D. The steeper the slope of the line (r), the higher the cervical resistance* ***(E)****.*

3.2.1.3. Preparation of trachea

Tracheas were dissected from non-pregnant rats which were in oestrous phase (160-260 g n=8), then blood vessels and the oesophagus were removed. The tracheal tube was sliced into 4-5 mm wide rings, then were placed in Krebs buffer (composition in mM: 118 NaCl; 4.75 KCl; 2.5 $CaCl_2$; 1.19 K_2HPO_4; 25 $NaHCO_3$; 1.2 $MgSO_4$ and 11 glucose). After the tracheal samples were installed with their longitudinal axis vertically by hooks, their initial strains were set to about 2.00 g.

3.2.2. Measurement of COX activity

The COX activity was measured in 22-day-pregnant and non-pregnant myometrial, tracheal, cervical and non-pregnant tracheal samples (n=6/group). After the preparation, the smooth muscle tissues were incubated in organ bath as described above. Then, they were perfused with cold Tris buffer pH 7.4 to clear away any red blood cells and clots, and were frozen in liquid nitrogen and stored at −80 °C until the measurement. On the day of assay, samples were homogenized in 5 ml of cold buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA) per gram tissue, centrifuged at 10.000 ×g for 15 min at 4 °C. The supernatant was stored on ice. The activity of COX enzymes was determined by COX Activity Assay Kit (Cayman Chemicals, Ann Arbor, MI) which measures the peroxidase activity of COX. The peroxidase activity is assayed with the colorimetric method by monitoring the appearance of oxidized *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm.

3.3. *In vivo* studies

The pregnant rats were split into four groups (n=8/group): (1) control, (2) tocopherol treated, (3) rofecoxib-treated, (4) tocopherol+rofecoxib-treated. The animals received a single treatment with 1 ml water (control), 250 mg/kg tocopherol[55], 5 mg/kg rofecoxib[56] or 250 mg/kg tocopherol+5 mg/kg rofecoxib on the 21st day of the pregnancy at 16:00 h by oral gavage. After the treatment, the onset of deliveries was detected and the elapsed hours were registered. Presence of blood or first foetus in the bedding was regarded as the onset of labour.

3.4. Statistical analyses

To the data were analysed by using Prism 5.01 (GraphPad Software, USA) computer program while the values were evaluated statistically with unpaired t-test and ANOVA Tukey-Multiple Comparison Test.

4. Results

4.1. Isolated organ bath studies

4.1.1. Uterus

4.1.1.1. Uterine contractions on non-pregnant and 22-day-pregnant uteri with or without tocopherol treatment

The KCl-evoked contraction of 22-day-pregnant uteri was higher than in non-pregnant uteri. In presence of tocopherol (10^{-7} M) the contraction of non-pregnant did not change while that of pregnant was increased significantly.

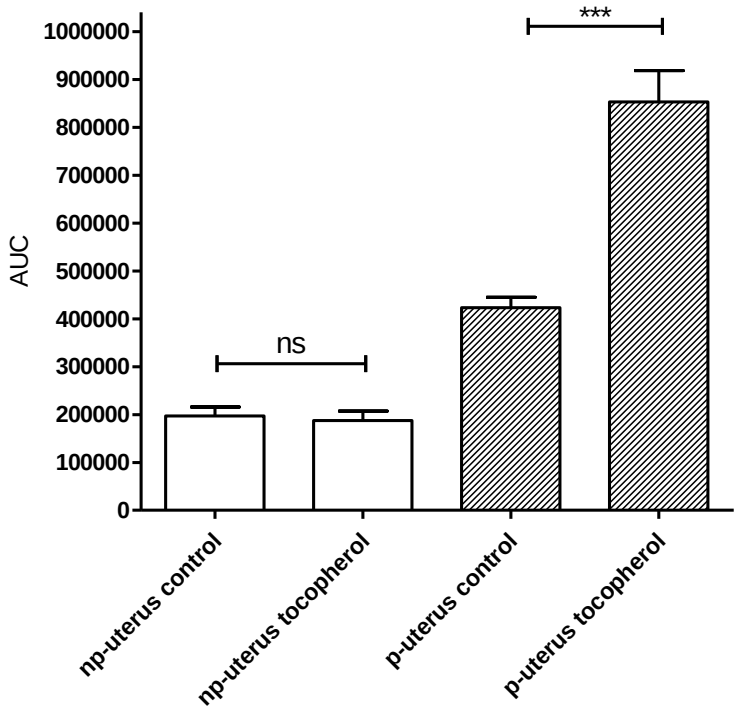


Figure 4. *Alteration of the uterine contractions (expressed in area under the curve - AUC) on non-pregnant* ***(empty columns)*** *and 22-day-pregnant* ***(striped columns)*** *uteri by incubation with tocopherol. The statistical analyses were carried out with the two-tailed unpaired t-test. (ns: not significant; *** $p < 0.001$) Each value denotes the mean ± S.E.M, n = 6.*

4.1.1.2. The effects of non-selective COX inhibitor diclofenac and selective COX-2 inhibitor rofecoxib on KCl-evoked control contraction of non-pregnant rat uteri alone and in the presence of tocopherol

The non-selective COX inhibitor diclofenac (10^{-9}–10^{-5} M) **(A)** and the selective COX-2 inhibitor rofecoxib (10^{-10}–10^{-5} M) **(B)** inhibited the contractions of non-pregnant uterus in a concentration-dependent manner. After tocopherol treatment, the relaxant effects of diclofenac and rofecoxib remained unchanged.

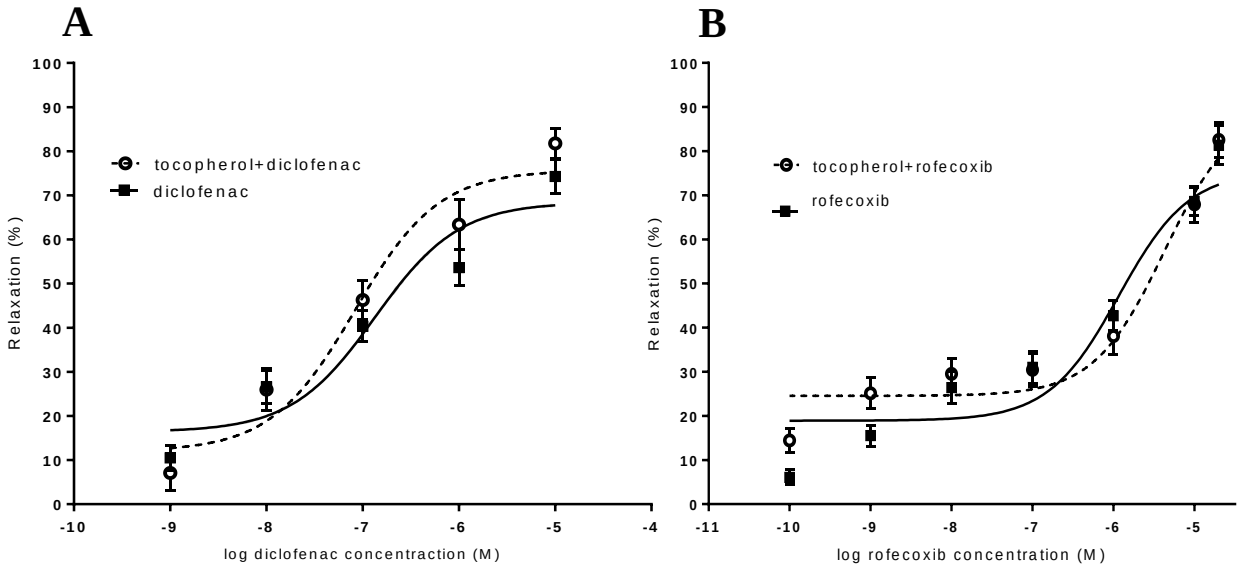


Figure 5. *The relaxant effect of non-selective COX inhibitor diclofenac (**A**) and selective COX-2 inhibitor rofecoxib (**B**) on non-pregnant rat uteri alone and in the presence of tocopherol (10^{-7} M). The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6.*

4.1.1.3. The effect of non-selective COX inhibitor diclofenac and selective COX-2 inhibitor rofecoxib on KCl-evoked control contraction of 22-day-pregnant rat uterus alone and in the presence of tocopherol

In the 22-day-pregnant uteri tissues, the maximum relaxant effect of selective COX-2 inhibitor rofecoxib was 55.33 %; it was 3.5 times as higher as than that of diclofenac (15.29 %). With tocopherol the impact of both compounds was enhanced significantly in each concentration.

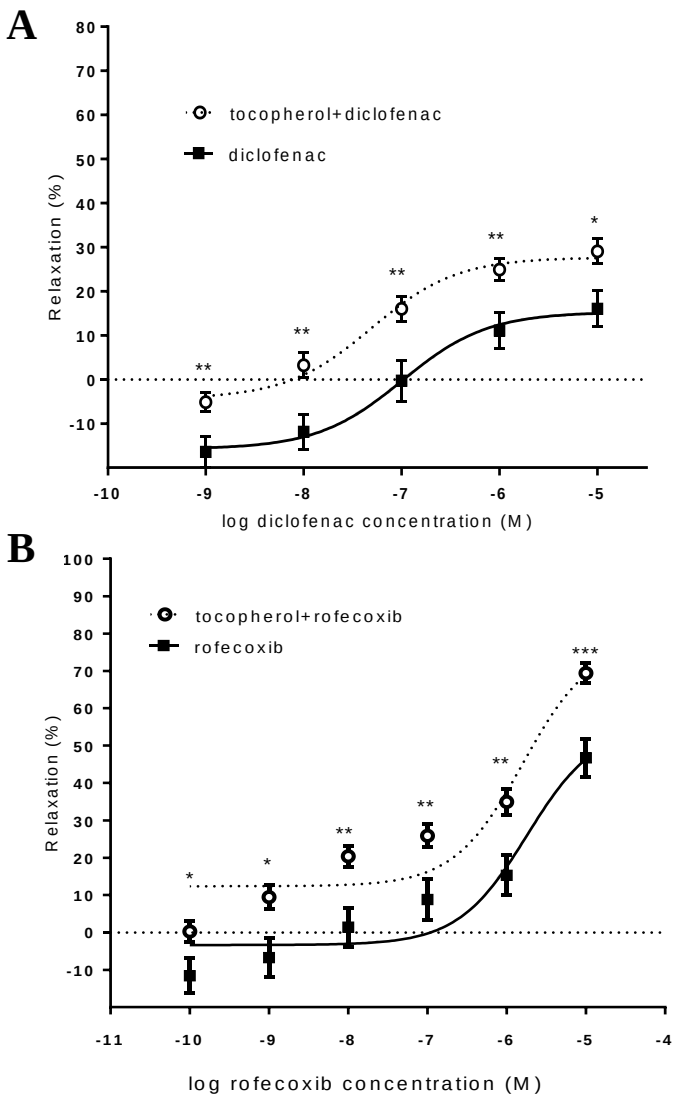


Figure 6. *The relaxant effect of nonselective COXi diclofenac (**A**) and selective COX-2 inhibitor rofecoxib (**B**) on 22-day-pregnant rat uteri alone and in the presence of tocopherol (10^{-7} M). The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6. (* p < 0.05; ** p < 0.01; *** p < 0.001)*

4.1.1.4. The effect of non-selective COX inhibitor diclofenac after pretreatment with selective COX-2 inhibitor rofecoxib on the KCl-evoked evoked contractions of 22-day-pregnant rat uteri alone and in the presence of tocopherol

When COX-2 was inhibited with one dose of rofecoxib (10^{-7} M) before the administration of diclofenac (10^{-9}–10^{-5} M), the relaxant effect of diclofenac was then practically ceased. **(continuous line)** The presence of tocopherol did not change this action. **(dotted line)**

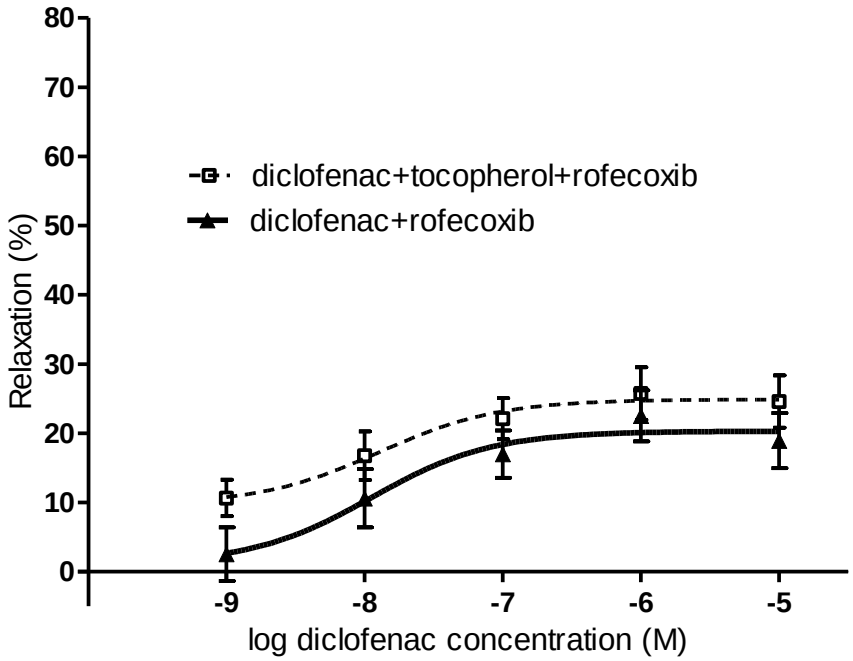


Figure 7. *The concentration-response curves of diclofenac after COX-2 inhibition with rofecoxib alone* ***(continuous line)*** *and in the presence of tocopherol* ***(dotted line)****. The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6.*

4.1.1.5. The effect of selective COX-2 inhibitor rofecoxib after pretreatment with selective COX-1 inhibitor SC-560 on the KCl-evoked evoked contractions of 22-day-pregnant rat uteri alone and in the presence of tocopherol

When COX-1 was inhibited with selective COX-1 inhibitor SC-560 (10^{-7} M) **(continuous line)**, the relaxing effect of rofecoxib was enhanced as compared with **Fig. 6/B** (concentration-response curve of rofecoxib). Tocopherol significantly increased further the uterine relaxant action of rofecoxib. **(dotted line)**

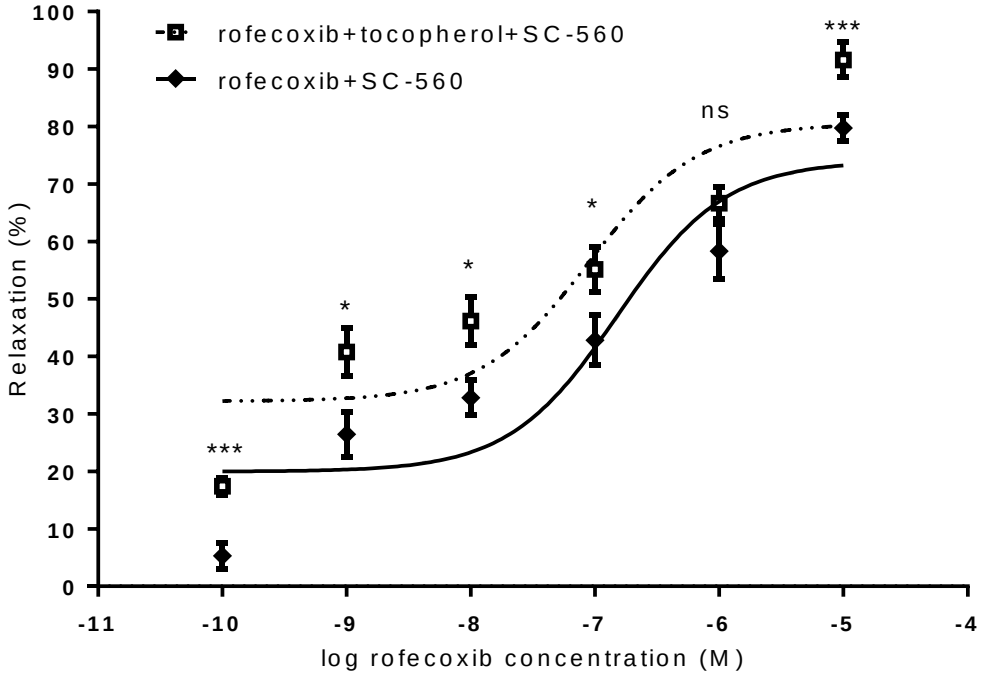


Figure 8. *The concentration-response curves of rofecoxib after COX-1 inhibition with SC-560 alone **(continuous line**) in the presence of tocopherol **(dotted line)**. The statistical analyses were carried out with the two-tailed unpaired t-test. (ns: not significant; *p < 0.05; *** p < 0.001) Each value denotes the mean ± S.E.M, n = 6.*

4.1.2. Trachea

4.1.2.1. Tracheal tone-reducing effect of non-selective COX inhibitor diclofenac and COX-2 selective rofecoxib on trachea tissues from rats in oestrus cycle

Both diclofenac (10^{-9}–10^{-5} M) and rofecoxib (10^{-10}–10^{-5}M) decreased the tone of tracheal samples. Diclofenac and rofecoxib reduced the average tone by 46.8 ± 5.0 mg and 32.6 ± 10.4 mg, respectively. Tocopherol impacted the effect of diclofenac and rofecoxib only in lower concentrations.

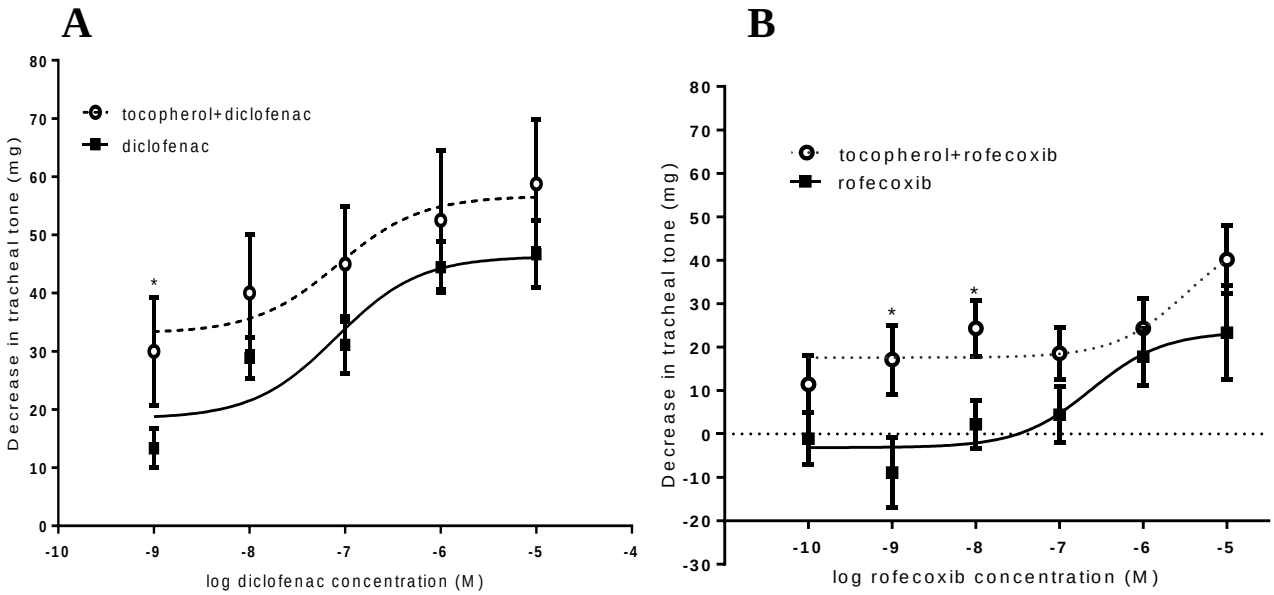


Figure 9. *Tone-reducing effect of non-selective COXi diclofenac (**A**) and selective COX-2 inhibitor rofecoxib (**B**) on trachea tissues from rats in oestrus cycle. The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6. (*p < 0.05)*

4.1.3. Cervix

4.1.3.1. The effects of alpha-tocopherol (10^{-7} M), COX inhibitors [diclofenac, SC-560, refocoxib] (10^{-6} M) and COX inhibitors combined with tocopherol in non-pregnant cervical resistance.

Neither the investigated COX inhibitors and tocopherol alone nor COX inhibitors combined with tocopherol altered the cervical resistance in non-pregnant cervical samples.

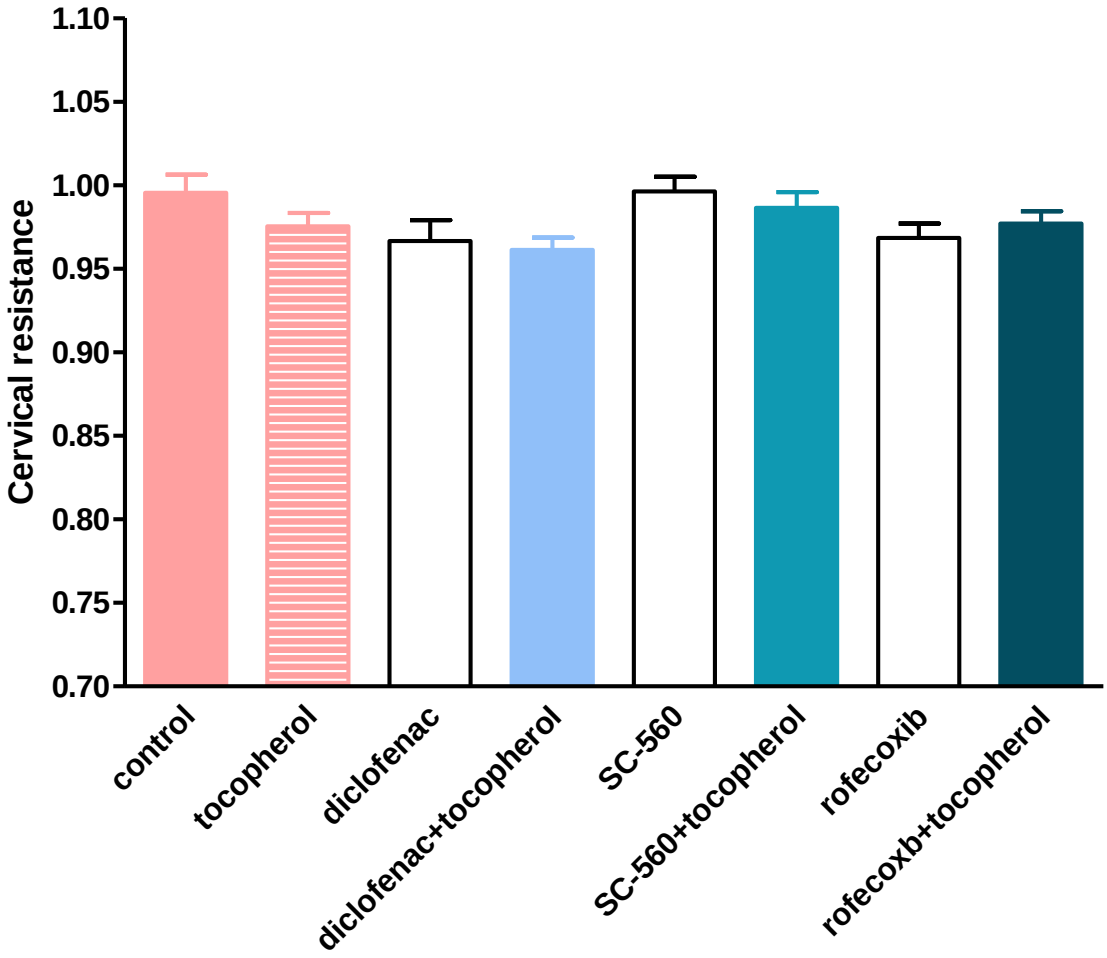


Figure 10. *Alteration of non-pregnant cervical resistance by tocopherol, different selectivity COX inhibitors alone and combined with tocopherol. The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6.*

4.1.3.2. The effects of alpha-tocopherol (10^{-7} M), COX inhibitors [diclofenac, SC-560, refocoxib] (10^{-6} M) and COX inhibitors combined with tocopherol in 22-day-pregnant cervical resistance.

In the 22-day-pregnant samples, the control cervical resistance was 0.85 ± 0.01. Tocopherol reduced resistance to 0.72 ± 0.02. The same remission in resistance (0.72 ± 0.02) was detected in the case of non-selective COX inhibitor diclofenac, and it was further decreased to 0.62 ± 0.02 by pre-treatment with tocopherol. The selective COX-1 inhibitor SC-560 possessed no effect on cervical resistance. However, in the presence of tocopherol, the resistance value was reduced to 0.69 ± 0.02. The selective COX-2 inhibitor rofecoxib also decreased cervical resistance to 0.70 ± 0.01. Rofecoxib with tocopherol induced the strongest reduction in cervical resistance (0.60 ± 0.02).

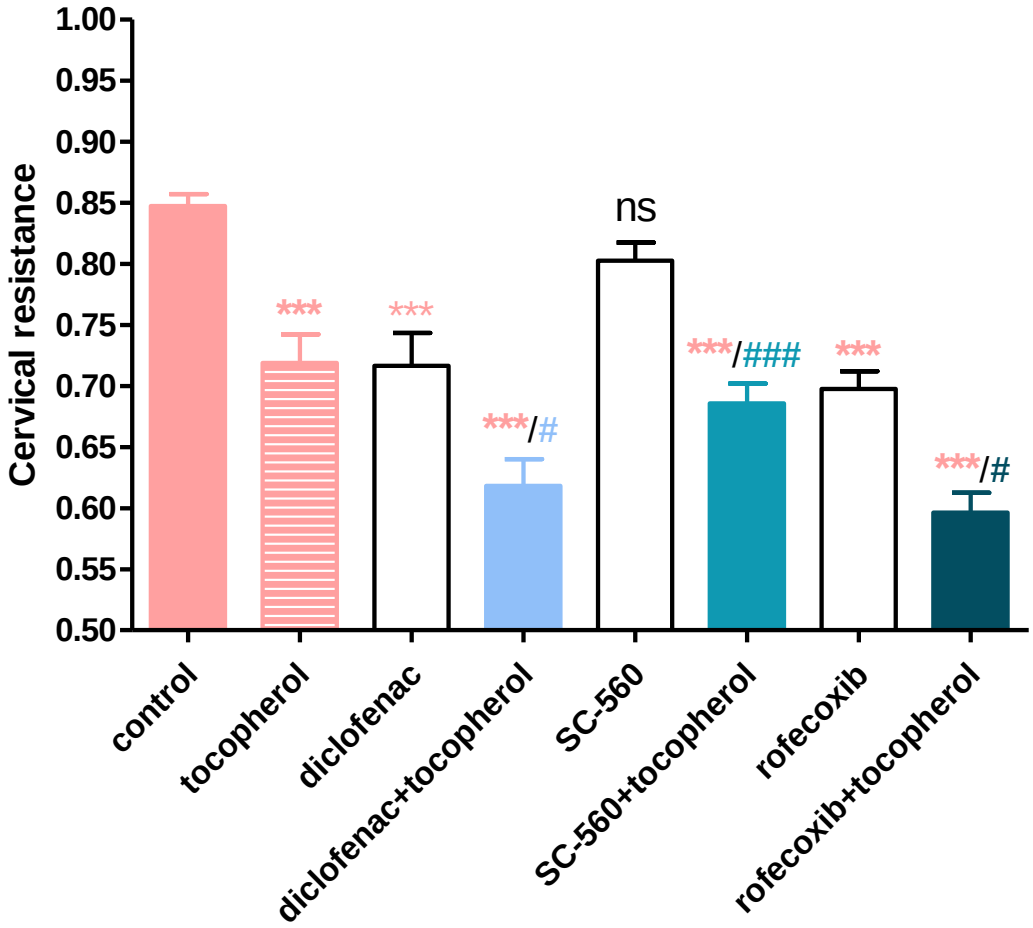
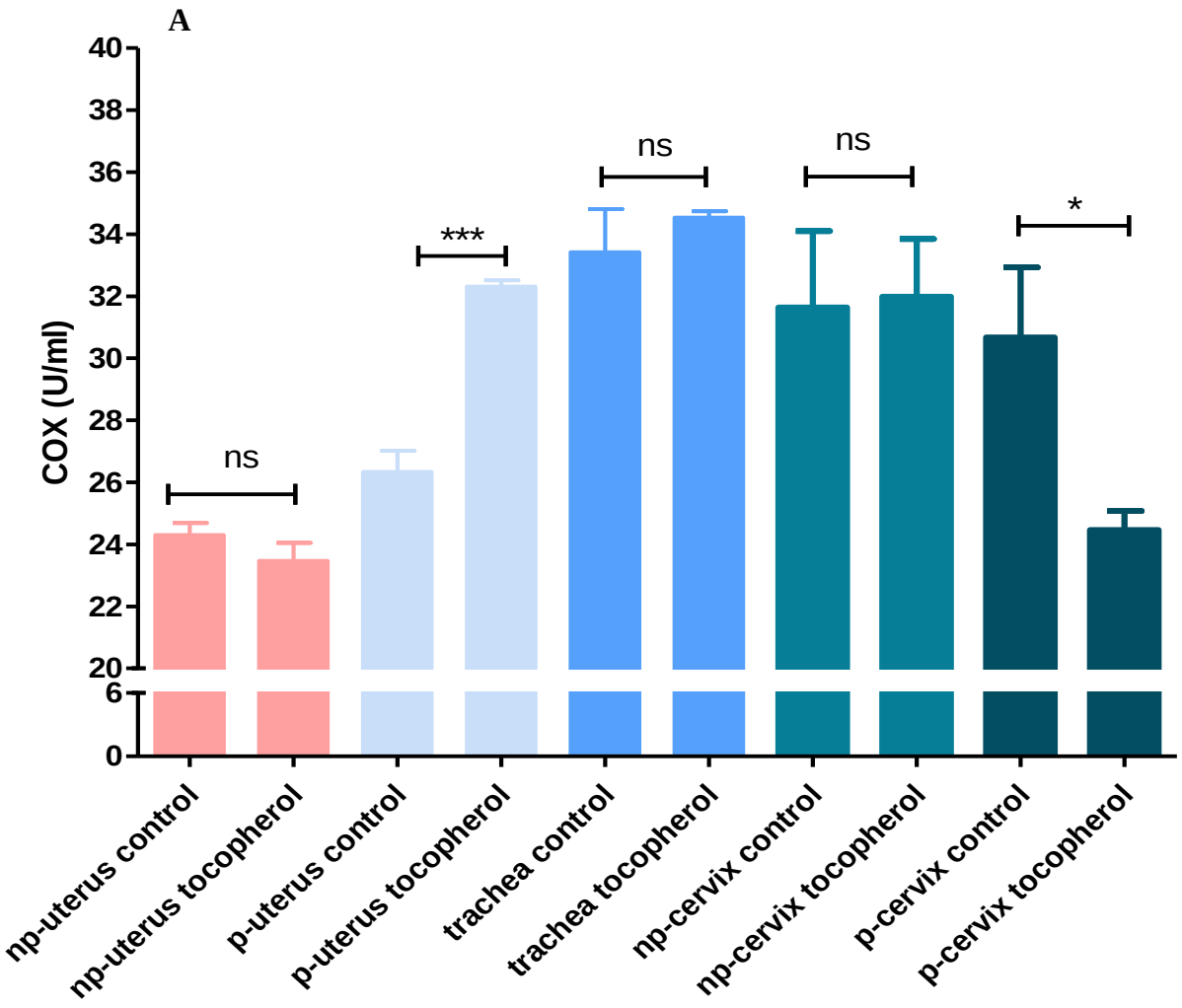


Figure 11. *Alteration of 22-day-pregnant cervical resistance by tocopherol, different selectivity COX inhibitors alone and combined with tocopherol. The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6. (ns: not significant; # p < 0.05; ***/### p < 0.001)*

4.2. COXs activities in the investigated smooth muscles tissues

The highest level of total COX activity was found in trachea tissues, while the lowest was in non-pregnant uteri. After pre-treatment with tocopherol, neither the COX activity of the trachea nor the COX activity of non-pregnant uteri and cervix changed. In 22-day-pregnant uterus and cervix, the total COXs activities were significantly increased by pre-treatment with tocopherol. The activity of COX-1 was not altered in the samples in the presence of tocopherol. However, the activity of COX-2 was enhanced significantly in tocopherol pre-treated 22-day-pregnant uterus and cervix.



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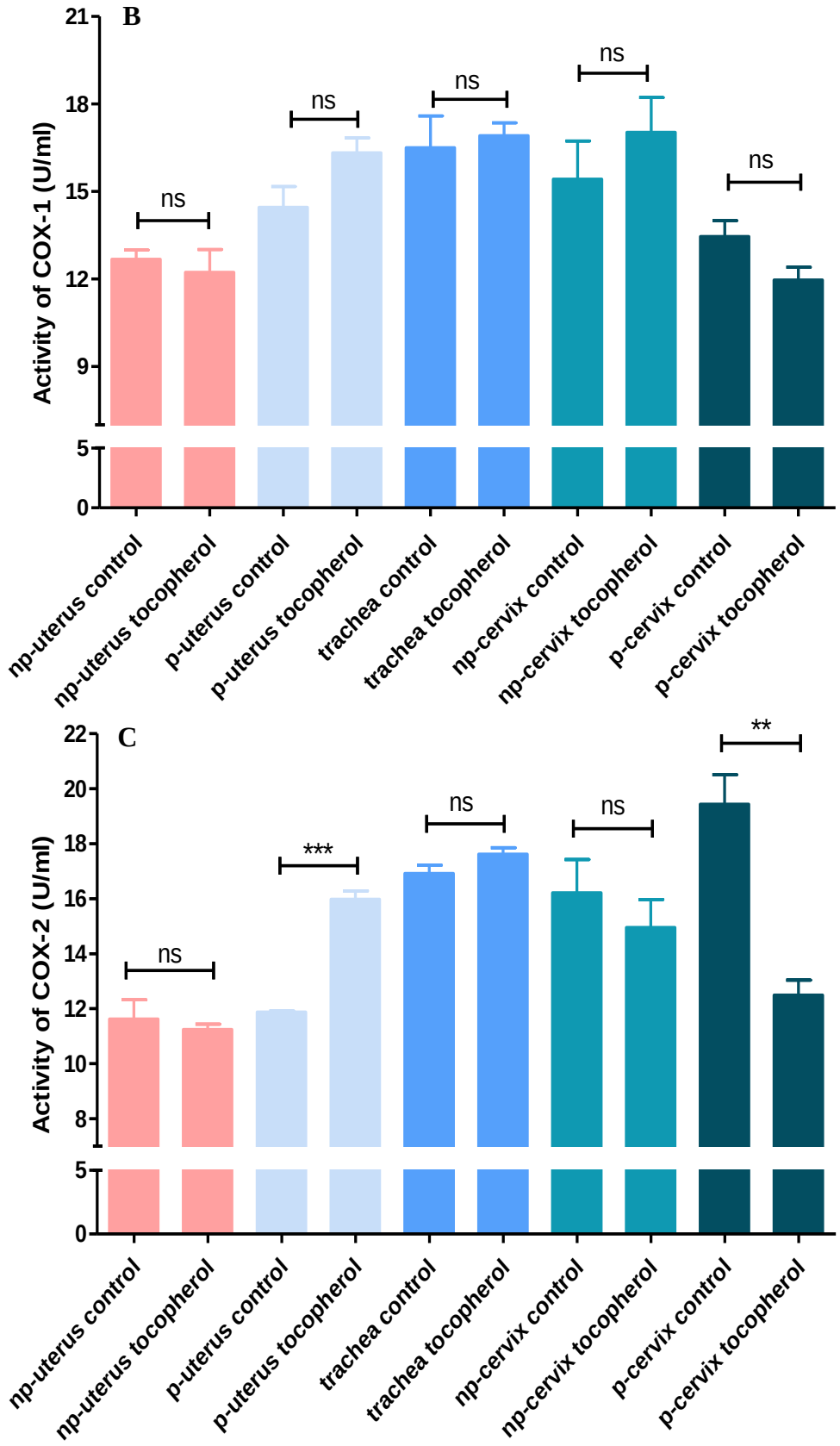

Figure 12. *Changes in the COXs activities in control and tocopherol treated tissues.* ***(A)****: total COXs activity,* ***(B)****: COX-1 activity,* ***(C)****: COX-2 activity*

*(np: non-pregnant, p: 22-day-pregnant) The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, n = 6. (ns:not significant; * p < 0.05; ** p <0.01; ***p < 0.001)*

4.3. *In vivo* studies

The delivery occurred 40 hours after the water treatment in control rats. Neither tocopherol- nor rofecoxib-treatment was able to change the time of delivery as compared with control groups. However, in case of co-administration of tocopherol and rofecoxib, the labour had been initiated 16 hours earlier as compared with the control group.

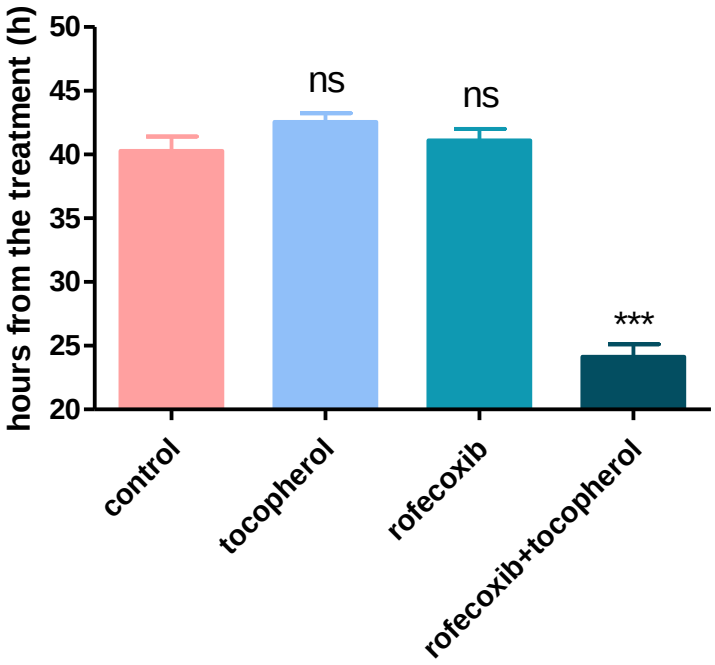

Figure 13. *Changes in the initiation time of delivery by tocopherol, rofecoxib and tocopherol combined with rofecoxib. The delivery occurred earlier by the treatment of tocopherol combined with rofecoxib. Each value denotes the mean ± S.E.M, n = 8. (ns: not significant; ***p < 0.001)*

5. Discussion

In this study, we demonstrated that α-tocopherol succinate modifies the uteri relaxant and cervical resistance-reducing effect of COX inhibitors via alteration of COX activity. Moreover, it was able to decrease the cervical resistance alone in 22-day-pregnant rats.

It is well known, that there are enormous differences between non-pregnant and pregnant female reproductive system. The whole reproductive tract undergoes a drastic transformation during pregnancy which is regulated by difficult biological mechanisms; this action is still not widely understood. The purpose of this alteration is to maintain the pregnancy and to prepare the mothers for labour at the end of pregnancy.

In pregnant uteri, the levels of several proteins and enzymes i.e.: PGs and COX enzymes are changed.[57–59] Moreover gap junctions and ion channels protein are activated in phase 1 of labor, while myometrial contractions are enhanced by oxytocin and prostaglandins in phase 2.[60]

PGs play an important role in uteri contractions and relaxations at the end of pregnancy. Thanks to the increased activity and expression of COXs, the levels of prostaglandins are enhanced. On the other hand, there is no general agreement in the literature as to which isoenzyme of COX leads to the liberation of prostaglandins at the time of parturition.

St-Louis et al.[61] suggested that the amounts of both COX-1 and COX-2 enzymes were equal in the oestrous phase in non-pregnant rat endometrium while in case of pregnant rat higher expression of COX-1 enzyme was observed than that of COX-2 at time of parturition. Other observations indicate that the expression of COX-2 was enhanced in rodent myometrium during labour, hence COX-2 derived PGs were determinant in the final pathway of parturition.[62] Furthermore, the increased expression of COX-2 issued in a high level of PGE_2, since the expression of COX-1 remained low and was not altered with gestational age in human uteri.[63] In our experiment, in non-pregnant uteri the activity of the COX-1 and COX-2 ratio was about similar, while in pregnant uteri the activity of COX-2 was temperately lower than that of COX-1 enzyme. In addition, the total COX activity of pregnant uteri was higher than in non-pregnant samples. The maximum effect of selective COX-2 inhibitor rofecoxib was more powerful than that of non-selective diclofenac both in pregnant and non-pregnant uteri. However, diclofenac as non-selective COX inhibitor is 20-fold more potent for COX-2 than COX-1.[64,65] These results indicate that both COX-1 and COX-2 enzymes play a role in uterine contractions, though, the

relaxation mostly depends on COX-2 inhibition. The relaxant effect of diclofenac and rofecoxib was more potent in non-pregnant than in pregnant uteri, which can be explained by the activities of COX-1 and COX-2 in these tissues. In non-pregnant uteri, the COX1/COX2 ratio is 1.09, which means that the two isoenzymes possess similar activity. Nevertheless, in pregnant uteri the COX-1 activity was 1.22, showing the greater activity of COX-1. Further evidence is that the amount of COX-2 determines the relaxing efficacy of COXi: less COX-2 means less efficacy, while more COX-2 means higher efficacy in the relaxing effect of COXi.

As in case of uteri, the functions and structures of non-pregnant and pregnant cervix are also different. The non-pregnant cervix is firm, it consists mostly of collagen in about 90 %, with a minority of smooth muscle (10 %). The pregnant cervix become dilated and softened, especially near term [66], thus the resistance of non-pregnant cervix is stronger than that of pregnant cervical resistance.[67] This phenomenon was clearly confirmed in our study. Prior the onset of labour, the cervix goes through the ripening process which is induced by numerous endogenous substances: PGs, cytokines and hormones such as progesterone, oestrogens and relaxin. Due to these compounds, at the term of pregnancy collagen is degraded and the cervix turns into hydrated, which causes the softness. [68] Some studies examined the exact role of PGs in cervical remodelling. PGE_2 enhanced the activity of collagenase in the human cervix [69] and the inflammatory response, which characterizes cervical ripening and remodelling.[70] PGF2α activated the synthesis of hyaluronic acid and glycosaminoglycans.[71] Moreover, prostaglandins were able to intensify cytokine synthesis[72] and PGE_2 stimulated cervical softening, while the non-selective COX inhibitor indomethacin inhibited this action.[73] However, those cervices were investigated on pregnancy day 17 and not on day 22, like in our experiment.

In the last decade, scientists investigated COX inhibitors as a probable tocolytic drugs. They established that the NSAIDs particularly indomethacin can decrease the contractility of uteri and inhibit the ripening of cervix.[74–76] On the other hand, just a few study are available which examined the effect of COX inhibitors on uteri or cervix near term. Klauser et al.[77] set against the tocolytic efficacy of magnesium sulphate, nifedipine and indomethacin in acute preterm women with 4-6 cm dilated cervix. Indomethacin could stop the preterm labour and extend the gestational period by 15.7 ± 20.6 days. Berghella et al.[78] found no significant benefit between indomethacin treated and control women who were in acute labour at gestational weeks of 14-25 with 1 cm dilated cervix. Furthermore, Marx et al. showed that in normal term pregnancy of rats the COX-2

enzyme was localized in cervical smooth muscle throughout gestation and its expression level was increased on 22-day labouring.[79] Kishore et al. demonstrated that the enhanced level of COX-2 enzyme and 15-PGDH down-regulation are crucial for the PGE2-induced cervical ripening.[80]

Based on these earlier findings, we presumed that COX inhibitors would enhance cervical resistance through abating the levels of PGs. Surprisingly, COX-1 selective inhibitor SC-560 possessed no action on cervical resistance, while the non-selective inhibitor diclofenac and COX-2 selective inhibitor rofecoxib lowered the pregnant cervical resistance.

Cervical remodelling is distributed into four different phases: softening, ripening, dilatation and postpartum. The dilatation phase is assigned directly before labour.[81] Our isolated organ bath experiments were carried out on pregnancy day 22. Since anticipated delivery of SD rats occurs on 22nd day of pregnancy, the cervical samples had probably undergone the ripening process. There is a new approach regarding the significance of cervical smooth muscle (CSM) in pregnancy. Over the past 60 years, the cervix has been known as a particularly collagenous structure.[82–84] Researchers have underrated the existence of smooth muscle in the cervix believed that CSM stays inactive in pregnancy and labour, and interpreted the premature cervical failure by the disorder of the cervical collagen network.[85–87] The CSM function has received more attention in the last years; [88] it was published that CSM stayed active during pregnancy and labour and in addition, it may contributed efficiently to cervical remodelling in rats. Vink et al. suggested that CSM has a possible role in uterine contraction and remodelling.[89] At the end of cervical ripening, collagens are degraded by matrix metalloproteinase, while the content of CSM remains fixed. Hence, it is possible that CSM may influence all phases of cervical remodelling and it might have a key function in the dilatation phase as well.

Furthermore, the differences in the selectivity of COX inhibitors imply that COXs may affect the dilatation of CSM. COXs catalyse the liberation of PGs in arachidonic acid cascade. In the cervix, both COX enzymes were observed, however, their amounts were different in non-pregnant and pregnant cervices. Dong et al. demonstrated that the cervical expression of COX-2 was elevated 2-fold at the end of pregnancy and the activity of COX-2 also rose during labour.[46] Therefore, COX-2 seems to be more essential for cervical ripening and spontaneous labour than COX-1. Although the activity of COX-2 did not change significantly in pregnancy, but it is visible that the COX-1/COX-2 ratio of pregnant cervices was shifted to direction of the predominance of COX-2. This result has

a discrepancy with earlier findings that proved the increased COX-2 expression in late-term rat [46], but in those experiment only the protein expressions of COXs were measured without detecting the real enzymatic activity.

Only a few studies investigated the relationship between vitamin E, its analogues and COXs.[48–50,52,53,90] Abate et al.[91] demonstrated that vitamin E reduced LPS-mediated COX-2 induction alone and in combination with aspirin in macrophages. Moreover, O'Leary et al. [92] reported that tocopherols decreased the activity of COX-2 in Caco2 cells. The drawback of those studies was that experiments were made in cell cultures, so their applicability is limited. At the same time, just a few papers focused on the connection of tocopherol and COXs in uteri and no research was carried out for the cervix before.

According to our results, pre-treatment with tocopherol unambiguously raised the area under the curve of KCl-evoked contractions in pregnant uteri. This implies that tocopherol may enhance the contractibility of pregnant myometrium. Interestingly, the influence of tocopherol was not detectable in the case of non-selective COX inhibitor diclofenac either in non-pregnant or in 22-day-pregnant uteri. These results suggest that, in contrast to earlier findings, pre-treatment with tocopherol may increase the contractility of uteri by enhancing the activity of COX-2 enzyme, and hereby the relaxant effect of COX inhibitors may be more pronounced, especially in the case of selective COX-2 inhibitors. To confirm this hypothesis, the alteration of the activity of COXs was measured in the tissues before and after the incubation with tocopherol. Tocopherol itself induced the COX activity and shifted the COX-1 and COX-2 ratio to COX-2 in pregnant uteri. When COX-1 was blocked by selective inhibitor SC-560, the relaxant effect of rofecoxib increased alone and further increased in the presence of tocopherol. However, when COX-2 was inhibited by the selective blocker rofecoxib, the dose-response curve of diclofenac was shifted slightly left, and after pre-treatment with tocopherol the significant difference between the curves practically ceased. These findings provide further evidence that COX-2 is predominant in pregnant uterine contraction and the tocopherol-induced modification of the COX-1 and COX-2 ratio led to the increased relaxing efficacy of COX inhibitors. In cervical samples, we found that tocopherol can reduce the pregnant cervical resistance and enhance the resistance-inhibition effect of diclofenac and rofecoxib. Its effect was maintained in the presence of COX-1 selective inhibitor SC-560. These results can be explained by COX activity measurements in which alpha-tocopherol decreased the activity of COX-2 in pregnant cervical samples. These findings suggest that COX-2 mediated PG liberation may have a crucial role in the contraction of CSM during

delivery. Moreover, tocopherol may have a synergist effect with COX inhibitors on rat cervical resistance.

The *in vivo* experiments implied that tocopherol together with COX-2 selective rofecoxib were able to shorten the gestational period by 16 hours. In the *in vitro* experiments we determined that they have a synergist effect on the reduction of cervical resistance. On the other hand, the co-administration of these compounds significantly reduced the myometrium contractions that would predict a delay in delivery. Thus, this *in vivo* result suggests that the joint effect of tocopherol and rofecoxib on the reduction of cervical resistance is predominant over their myometrium relaxing effect.

Finally, in trachea tissues the levels of COX-1 and COX-2 activity were similar (the COX-1/COX-2 ratio was 0.97), which correlates with the previous findings in literature.[93,94] Diclofenac decreased the tracheal tone more than rofecoxib, suggesting that COX-1 derived prostaglandins may play a larger role in airway smooth muscle contraction in rat. Interestingly, pre-treatment with tocopherol increased the relaxing effects of rofecoxib and diclofenac, but only in low concentrations of the drugs. We have no clear explanation for this phenomenon, but it might be related to the weak antioxidant capacity of rat trachea as compared with pregnant uterus.[24]

6. Conclusion

The mechanism of action of vitamin E is extremely complex. It is known that it can influence the activity of various receptors and enzymes but in many cases its exact process is unfamiliar.

In the light of our results, we can conclude that:

1. COX enzymes, especially COX-2 and PGs liberated by COX-2 play a significant role in the contraction of pregnant uteri and in cervical ripening, and also in the contraction of cervical smooth muscle before parturition.

2. Tocopherol can strengthen COX-2 activity in pregnant uteri, leading to the stronger relaxant effect of COX-2 inhibitor; at the same time it has no such action in non-pregnant uteri or tracheal tissue.

3. Alpha-tocopherol potentiates the cervical-resistance reducing effect of COX inhibitors via the inhibition of COX-2 activity in late-term and ripened rat cervix. Interestingly, alpha-tocopherol has an opposite effect on COX-2 activity in pregnant cervices as compared with pregnant myometria. Hereby, it seems that tocopherol has a tissue specific COX-2 activity effect.

4. The single oral administration with tocopherol and rofecoxib can shorten the gestational period and accelerate the onset of labour. This result suggests that this synergist effect between tocopherol and rofecoxib on the reduction of cervical resistance is prevailing over their myometrial relaxing effect.

Our results contribute to the understanding of the complex mechanism of action of vitamin E revealing its influence on the COX system. Although the experiments were carried out with rats, the data may provide a basis for further studies in humans.

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8. List of publications

8.1. Publication related to Ph.D. thesis

I. Kothencz A., Hajagos-Tóth J., Csányi A., Gáspár R.: Alpha-tocopherol succinate increases cyclooxygenase-2 activity: Tissue-specific action in pregnant rat uterus in vitro.
Life Sciences 192 pp. 199-204. 6 p. (2018)
[IF: 3.448; D1 in Pharmacology, Toxicology and Pharmaceutics (miscellaneous) (2018)]

II. Kothencz A., Hajagos-Tóth J., Szűcs K. F.; Schaffer A., Gáspár R.: α-Tocopherol Potentiates the cervical resistance decreasing effects of COX inhibitors in pregnant rats: the putative role of Cyclooxygenase-2 inhibition.
Journal of Pharmacology and Experimental Therapeutics 368: 2 pp. 292-298. 7 p. (2019)
[IF: 3.615; Q1 in Pharmacology (2018)]

8.2. Presentation related to Ph.D. thesis

I. Kothencz A., Hajagos-Tóth J., Gáspár R.
The antioxidant α-tocopherol modifies the smooth muscle effects of NSAIDs
RECOOP 12th Bridges in Life Sciences Annual Conference, Budapest, Hungary, 2017 (Poster presentation)

II. Kothencz A., Hajagos-Tóth J., Gáspár R.
Interaction of alpha-tocopherol and cyclooxygenase-inhibitors on smooth muscles of rats: the significance of cyclooxygenase-activity in uterus and trachea
FEPS Congress Vienna, Austria, 2017 (Poster presentation)

III. Kothencz A., Hajagos-Tóth J., Gáspár R.
Az antioxidáns alfa-tokoferol módosítja a nem szteroid gyulladásgátlók simaizomra gyakorolt hatását
XX. Tavaszi Szél Konferencia Nemzetközi Multidiszciplináris Konferencia, Miskolc, Hungary, 2017 (Oral presentation)

IV. Kothencz A., Hajagos-Tóth J., Gáspár R.

Az alfa-tokoferol és COX-gátlók cervix rezisztenciára gyakorolt hatásának vizsgálata

XXI. Tavaszi Szél Konferencia Nemzetközi Multidiszciplináris Konferencia, Győr, Hungary, 2018 (Oral presentation)

V. Kothencz A., Hajagos-Tóth J., Gáspár R.

Changes in the cervical resistance by COX-inhibitors and alpha-tocopherol in rat

RECOOP 13th Bridges in Life Sciences Annual Conference, Zagreb, Croatia, 2018 (Poster presentation)

VI. **Kothencz A.**, Hajagos-Tóth J., Szűcs K. F., Schaffer A., Gáspár R.

Alpha-tocopherol modifies the smooth muscle relaxant and cervical resistance effect of COX inhibitors in rats.

Euro summit on Toxicology and Pharmacology Rome, Italy, 2019 (Oral presentation)

8.3. Other publication nonrelated to this thesis

I. Szűcs KF, Grósz Gy., Süle., M., Sztojkov-Ivanov A., Ducza E., Márki Á., **Kothencz A.**, Balogh L., Gáspár R.: Detection of stress and the effects of central nervous system depressants by gastrointestinal smooth muscle electromyography in wakeful rats.

Life Sciences 205 pp. 1-8.,8 p. (2018) **[IF: 3.448; D1 in Pharmacology, Toxicology and Pharmaceutics (miscellaneous) (2018)]**

II. Csányi A., Hajagos-Tóth J., **Kothencz A.,** Gáspár R., Ducza E.: Effects of different antibiotics on the uterine contraction and the expression of aquaporin 5 in term pregnant rat.

Reproductive Toxicology 81 pp. 64-70., 7 p. (2018) **[IF: 3.200; Q2 in Toxicology (2018)]**

III. Zoofishan Z., Kúsz, N., Csorba, A., Tóth, G., Hajagos-Tóth, J., **Kothencz A.**, Gáspár, R., Hunyadi A.: Antispasmodic Activity of Prenylated Phenolic Compounds from the Root Bark of Morus nigra.

Molecules 24: 13 Paper: 2497 (2019) **[IF: 3.060; Q1 in Chemistry (miscellaneous) (2018)]**

IV. Ducza E, Csányi A, Szőke É, Pohóczky K, Hajagos-Tóth J, **Kothencz A**, Tiszai Z, Gáspár R: Significance of transient receptor potential vanilloid 4 and AQP 5 co-expression in the rat uterus at term.

Heliyon 5: 10 p. Paper: 02697 (2019) **[IF:-; Q1 in Multidisciplinary]**

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