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Faculty of Pharmacy

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The influence of alpha-tocopherol on the smooth muscle contractions: the significance of cyclooxygenase function

Ph.D. Thesis Summary

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Introduction

In 1922, Evans and Bishops published the first article about the discovery of vitamin E, which is crucial for the normal physiological functions. In the nature, vitamin E exists as a mixture of eight forms such as α-, β-, γ-, δ-tocopherols, and tocotrienols. However, the activity of these analogues is quite various, it seems that α-tocopherol is the most effective against the vitamin E deficiency disease and it can reach the highest plasma concentration in the human body.

The mechanism of action of vitamin E is extremely complex. 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. Moreover, beyond of the protection, tocopherol participates in the action of redox regulated enzymes and transcriptions factors. 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.

Vitamin E plays a significant role in pregnancy, the enhanced plasma level of α-tocopherol protects foetus, placenta and uterus from oxidative damage. It is also known, that vitamin E possesses an anti-inflammatory ability thus, in the last decade it has been suggested that vitamin E may help the treatment or prevention of asthma.

In the arachidonic acid cascade, 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. During the transformation, due to peroxidase activity of COXs, reactive oxygen species (ROS) can be released.

The liberated PGs play an important role in contraction or relaxation of smooth muscles and cervical ripening during pregnancy.

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. COX inhibitors relax smooth muscles via diminish the production and release of prostaglandins. Thus, in clinical practise they are used for preterm therapy till 35 weeks of pregnancy. So far, only a few studies are published about the association of vitamin E and COXs, in addition their results are inconsistent.

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 this 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 this study was to analyse how the 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 alone and in combination change the time of the initiation of parturition in rats *in vivo*.

Materials and methods

All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission number: IV/198/2013).

Mature female (180-200 g) and male (240-260 g) Sprague-Dawley rats were mated in a special mating cage. Vaginal smears sample were taken from the female rats and a sperm search was performed under a microscope at a magnification of 1200x. If there was copulation plug or the vaginal smears were positive the female rats were separated as first-day pregnant animals.

***In vitro* studies**

22-day-pregnant and non-pregnant rats in oestrous phase were used for *in vitro* 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 and recorded with a SPEL Advanced ISOSYS Data Acquisition System (MDE Ltd., Budapest, Hungary). Tissues were incubated for 1 h with a buffer renewal in every 15 minutes. Then samples were equilibrated for another 60 min with α-tocopherol succinate (10^{-7} M); it was added to samples after every wash of buffer solution. The control preparations were incubated for 1 h without tocopherol.

Preparation of uteri

After the termination, uterus samples were cut into 5-mm long muscle rings, were mounted vertically in an organ baths containing 10 ml de Jong buffer and their initial tension 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.

Preparation of trachea

Tracheas were dissected from non-pregnant rats. The tracheal tube was sliced into 4-5 mm wide rings and then was placed in Krebs buffer. After the tracheal samples were installed with their longitudinal axis vertically by hooks, their initial strains were set to about 2.00 g. The tracheal tone reducing effect of non-selective COXi diclofenac (10^{-9}–10^{-5} M) and COX-2 selective inhibitor rofecoxib (10^{-10}–10^{-5} M) were obtained.

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 to setting the initial tension 1.00 g, cervices were equilibrated with mentioned method. The cervices were stretched in growing steps and allowed to relax for 5 min. After every 5 min, the next initial tension was set, in 1-g steps between 1 and 12 g. 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.

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, frozen in liquid nitrogen and stored at −80 °C until the measurement. On the day of assay, samples were homogenized cold buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA), 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.

***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, 5 mg/kg rofecoxib or 250 mg/kg tocopherol+5 mg/kg rofecoxib on the 21 day of 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.

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.

Results

Results of *in vitro* studies

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. **(Fig. 1.)**

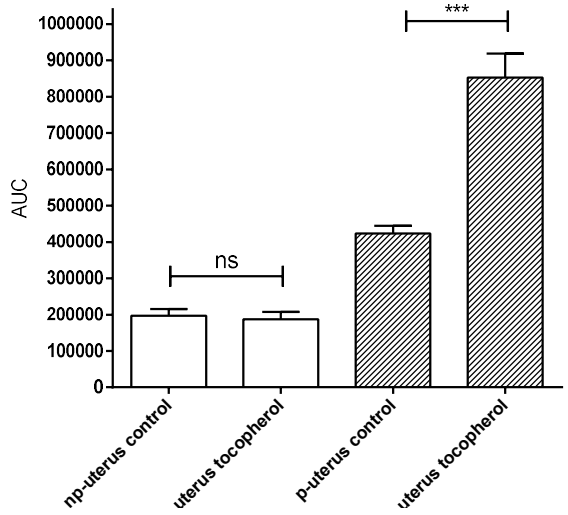

Fig. 1: Alteration of the uterine contractions (expressed in area under the curve - AUC) on non-pregnant **(empty columns)** and 22-day-pregnant **(striped columns)** uteri by incubation with tocopherol. (ns > 0.05; *** $p < 0.001$)

The non-selective COX inhibitor diclofenac (10^{-9}–10^{-5} M) **(Fig. 2/a)** and the selective COX-2 inhibitor rofecoxib (10^{-10}–10^{-5} M) **(Fig. 2/b)** inhibited the contractions of non-pregnant uterus in a concentration-dependent manner. After tocopherol (10^{-7} M) treatment, the relaxant effects of diclofenac and rofecoxib remained unchanged.

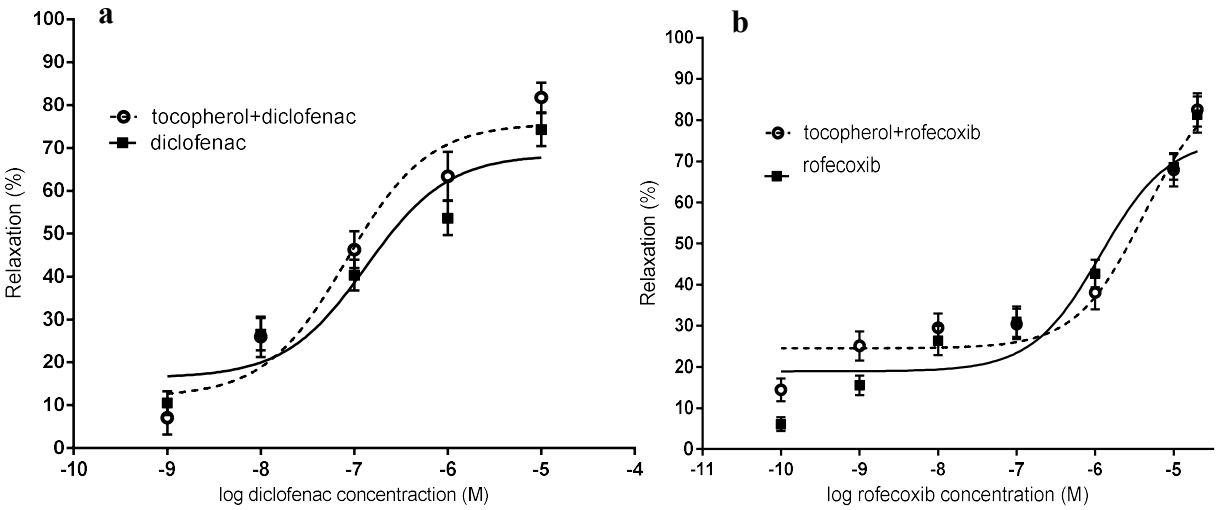

Fig. 2: The relaxant effect of non-selective COX inhibitor diclofenac (**a**) and selective COX-2 inhibitor rofecoxib (**b**) on non-pregnant rat uteri alone and in the presence of tocopherol.

In the 22-day-pregnant uteri tissues, the maximum relaxant effect of selective COX-2 inhibitor rofecoxib **(Fig. 3/b)** was 55.33 %; it was 3.5 times as higher as than that of diclofenac **(Fig. 3/a)**; (15.29 %). With tocopherol the impact of both compounds was enhanced significantly in each concentration.

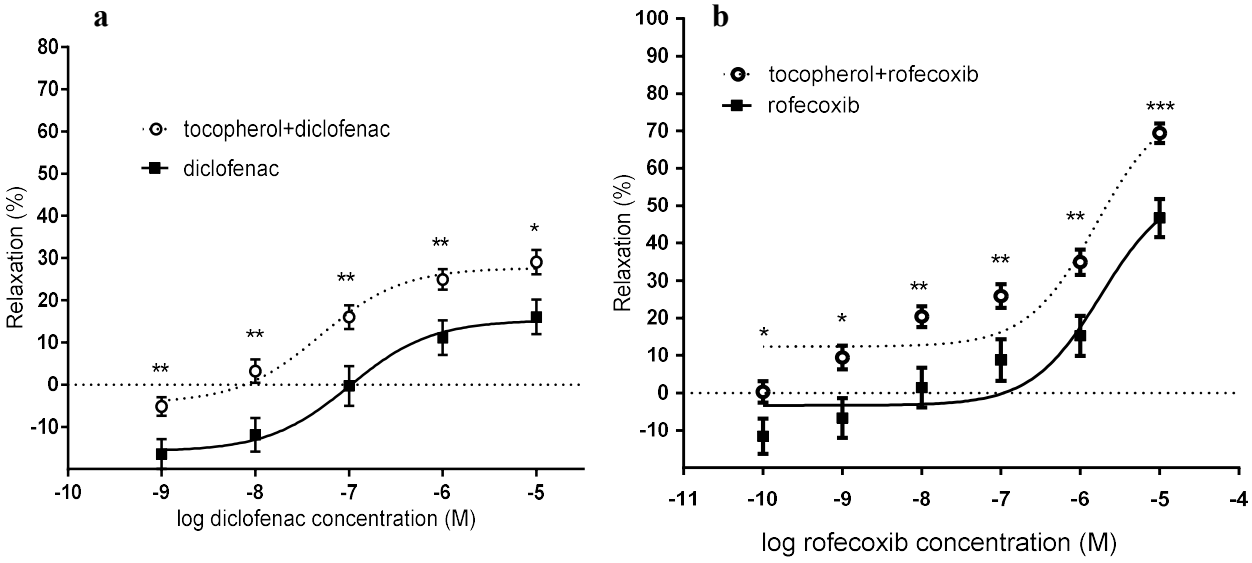

Fig. 3: The relaxant effect of nonselective COXi diclofenac (**a**) and selective COX-2 inhibitor rofecoxib (**b**) on 22-day-pregnant rat uteri alone and in the presence of tocopherol (10^{-7} M). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

When COX-2 was inhibited with one dose of rofecoxib (10^{-7} M) before the administration of diclofenac (10^{-9}–10^{-5} M), then the relaxant effect of diclofenac was practically ceased. **(continuous line)** The presence of tocopherol did not change this action. **(dotted line) (Fig. 4)**

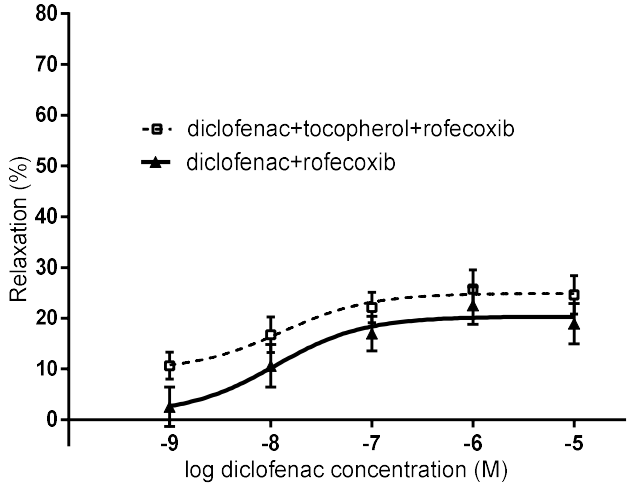

Fig. 4: The concentration-response curves of diclofenac after COX-2 inhibition with rofecoxib alone **(continuous line)** and in the presence of tocopherol **(dotted line)**.

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. 8/B** (concentration-response curve of rofecoxib). The tocopherol significantly increased further the uterine relaxant action of rofecoxib. **(dotted line) (Fig. 5)**

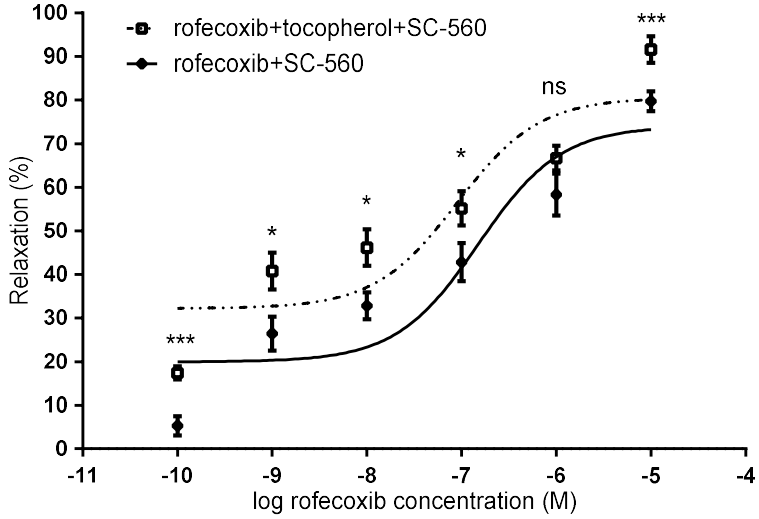

Fig. 5: The concentration-response curves of rofecoxib after COX-1 inhibition with SC-560 alone **(continuous line)** in the presence of tocopherol **(dotted line).** (ns > 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001)

Both diclofenac (10^{-9}–10^{-5} M) **(Fig. 6/a)** and rofecoxib (10^{-10}–10^{-5}M) **(Fig. 6/b)** 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

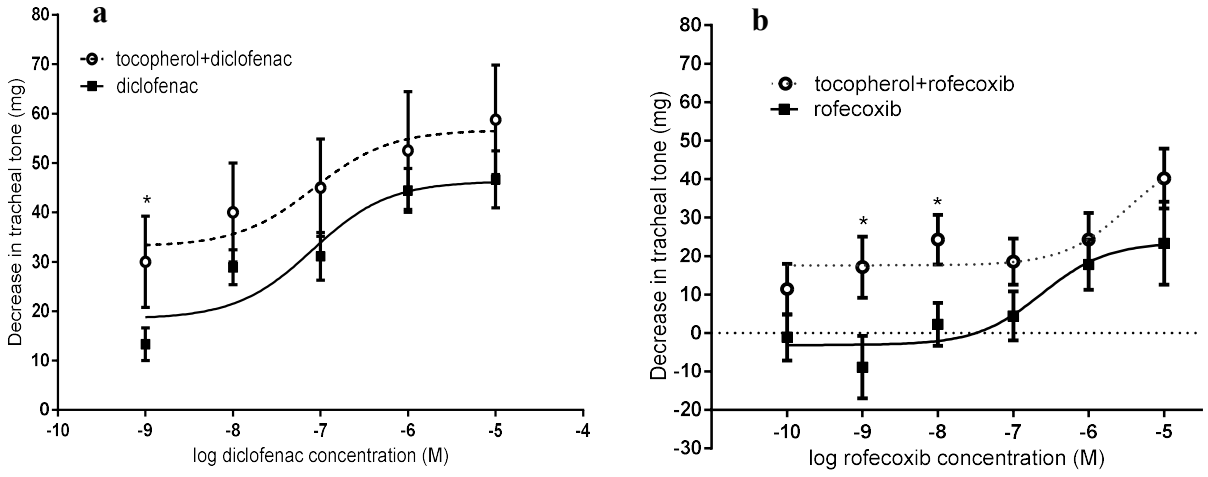

Fig. 6: Tone-reducing effect of non-selective COXi diclofenac (**a**) and selective COX-2 inhibitor rofecoxib (**b**) on trachea tissues. (ns > 0.05; * p < 0.05)

Neither the investigated COX inhibitors and tocopherol alone nor COX inhibitors combined tocopherol altered the cervical resistance in non-pregnant cervical samples. **(Fig. 7)**

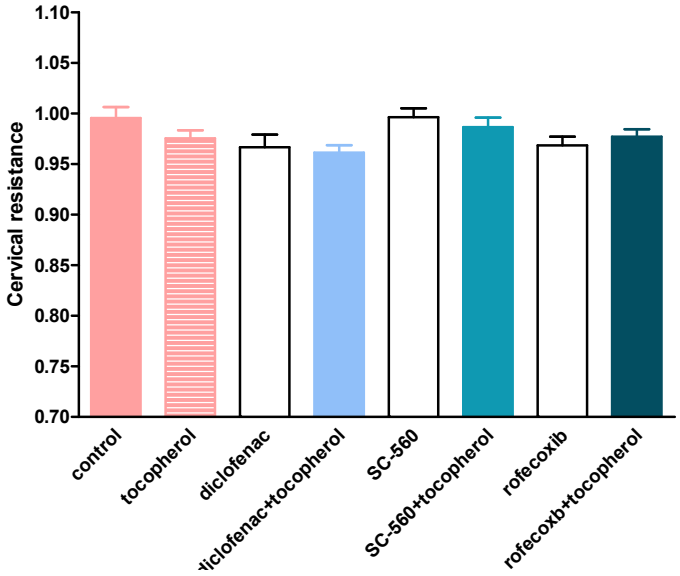

Fig. 7: Alteration of non-pregnant cervical resistance by tocopherol, different selectivity COX inhibitors alone and combined with tocopherol.

In the 22-day-pregnant samples, the control cervical resistance was 0.85 ± 0.01. Alone the α-tocopherol, selective COXi diclofenac and selective COXi rofecoxib reduced the resistance. The selective COX-1 inhibitor SC-560 possessed no effect on cervical resistance. On the other hand, α-tocopherol combined with COX inhibitors, decreased further the resistance in all cases. **(Fig. 8)**

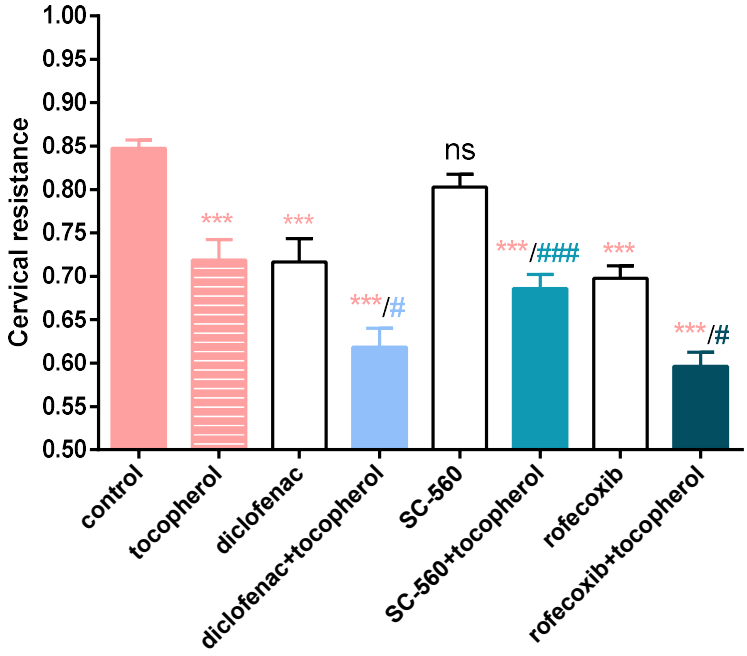

Fig. 8: Alteration of 22-day-pregnant cervical resistance by tocopherol, different selectivity COX inhibitors alone and combined with tocopherol. (ns > 0.05; # $p < 0.05$; ***/### $p < 0.001$)

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. **(Fig. 9)**

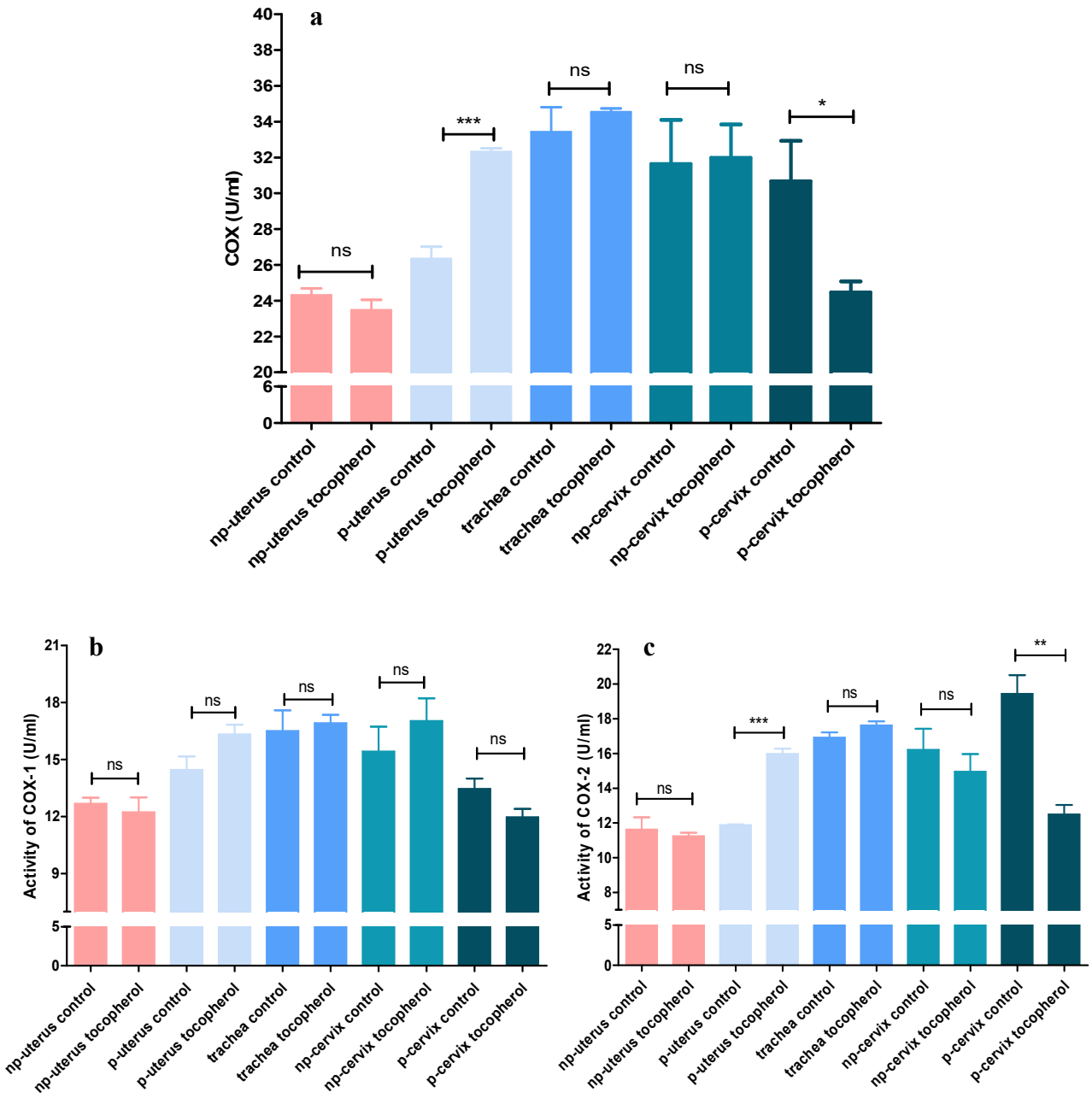

Fig. 9: Changes in the COXs activities in control and tocopherol treated tissues. **(a):** total COXs activity, **(b):** COX-1 activity, **(c):** COX-2 activity (np: non-pregnant, p: 22-day-pregnant); (ns > 0.05; * p < 0.05; ** p <0.01; ***p < 0.001)

Results of *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. **(Fig. 10)**

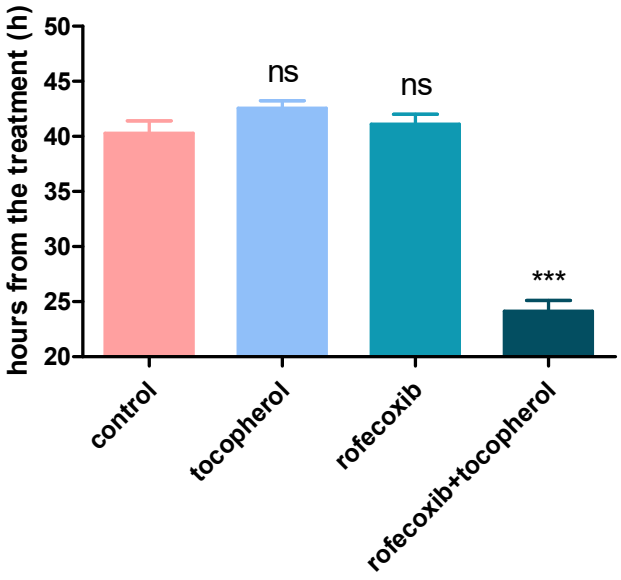

Fig. 10: Changes the initiation time of delivery by tocopherol, rofecoxib and tocopherol combined with rofecoxib. (ns > 0.05; ***p < 0.001)

Discussion

The whole reproductive tract undergoes a drastic transformation during pregnancy which is regulated by difficult biological mechanisms; this action is still not widely understood. PGs play an important role in uteri contractions, relaxations and also in cervical ripening at the end of pregnancy.

During pregnancy, the cervix goes through the ripening process which is induced by numerous endogenous substances such as PGs. The pregnant cervix become dilated and softened, especially near term, thus the resistance of non-pregnant cervix is stronger than that of pregnant cervical resistance. This phenomenon was clearly confirmed in our study. 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.

Over the past 60 years, the cervix has been known as a particularly collagenous structure. Researchers have underrated the existence of smooth muscle in the cervix believed that (cervical smooth muscle) CSM stays inactive in pregnancy and labour, and interpreted the premature cervical failure by the disorder of the cervical collagen network. The CSM function has received more attention in the last years, it stayed active during pregnancy and labour in addition, it may contributed efficiently to cervical remodelling in rats. Moreover, it has a possible role in uterine contraction. 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. 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. Based on these facts, we presumed that the differences in the selectivity of COX inhibitors imply that COXs may affect the dilatation of CSM.

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. These results suggest that 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 which correlates with the previous findings in literature. 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.

Conclusion

In the light of our results, we can conclude that α-tocopherol has a tissue specific COX activity modifying action thus, it can influence the effect of COX inhibitors. In pregnant uteri tocopherol can strengthen COX-2 activity, leading to the stronger relaxant effect of COX-2 inhibitor. On the other hand, α-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. Finally, 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 myometrium relaxing effect.

Appendix

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 recreasing 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)]**

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)

3. Other publication unrelated 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; [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]**