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[Inhibition of arachidonic acid cascade by extract of rye pollen]

[Article in German]

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A standardized extract mainly from rye pollen (Cernilton N) was tested in vitro on the inhibition of prostaglandin and leukotrien synthesis. The determination of the prostaglandin and leukotrien synthesis from labelled arachidonic acid was done in microsomes of ram seminal vesicles resp. in rat basophilic leukemia cells (RBL-1 cells). The water soluble resp. the fat soluble extract fraction from the whole pollen extract were tested separately. The radio-TLC separation of the reaction metabolites showed a dose dependent inhibition of the cyclo-oxygenase and the 5-lipoxygenase activity by the fat soluble pollen extract fraction. The IC50-values are 0.005 mg/ml resp. 0.08 mg/ml and similar to those of the also tested diclofenac. The water soluble fractions showed no effect in this test system. According to these in vitro results and the clinical experience so far with the pollen extract its therapeutic efficacy on benign prostate diseases is best explainable by the anticongestive resp. anti-inflammatory effect of the fat soluble fraction. Due to the different actions of prostaglandins and leucotrienes also relaxant and antiproliferative effects were conceivable.

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Inhibition of the Arachidonic Acid Metabolism by an Extract from Rye Pollen

G. Loschen, L. Ebeling

Introduction

Clinical studies with a defined pollen extract preparation document its symptomatic efficacy in patients with benign prostatic diseases. In patients with benign prostatic hyperplasia (BPH) a significant reduction in nocturia and residual urine is observed (4,8). The continued improvement in symptoms (5) and a significant reduction in the anteriorposterior diameter of the prostate after six months of treatment (8) suggest a permanent pharmacological influence on pathophysiological alterations induced by the underlying disease. In patients with chronic non-bacterial prostatitis a significant improvement in symptoms or even a symptom-free status is achieved as it is in patients with prostatodynia. Furthermore, a reduction or even normalization of the white cell count in prostatic secretions has been documented (7).

Despite the fact that prostaglandins have been demonstrated for the first time in the prostate, and despite the fact that the entire group of substances received its name based on their increased presence in the prostate gland (10), little is known as of yet about their function in this particular organ. What is known, however, is that prostaglandins and leukotriens play an important role in inflammatory reactions (11). Furthermore, an etiologic role in the development of BPH has been suggested (1).

The majority of the known mediators of inflammation arise from the membranebound arachidonic acid. Their intracellular release by activation of phospholipases facilitates their enzymatic metabolism in a cascade of pharmocologically very potent reacation products. The biosynthesis of the eicosanoid-derived inflammatory mediators, which according to present pharmacological knowledge are of importance for the understanding of the pathologic alterations on a molecular level, is initiated by two enzymes: cyclo-oxygenase and 5-lipoxygenase.

The therapeutic effectiveness of many drugs can be explained by their interactions with enzyms that are responsible for individual steps in the metabolism of arachidonic acid (11). The clinical effectiveness of the pollen extract in benign prostatic diseases therefore leads to the question, whether, and to what extent this extract influences the biosynthesis of prostaglandins and leukotriens in vitro.

Material

The examined pollen extract1 is produced by AB Cernelle, Engelholm (Sweden). It consists predominantly (greater than 90 %) of rye pollen (Secale cereale L.) as well as two other quantitatively relatively unimportant types of pollen. The exact composition can be obtained from the manufacturer. The pollen is extracted initially with water and thereafter with acetone. For the experiment discussed herein the water-soluble (wPE) and fatsoluble (fPE) fractions which were standardized for their content in a-amino-acids (18.3 % w/w) and for phytosterols (1.1 % w/w) were tested separately. The experiments were conducted in the research laboratories of Grünenthal GmbH, Aachen, Germany.

14C-marked arachidonic acid and the radioactive-marked metabolites of arachidonic acid (PGF2; PGE2; LTB4; 5-HETE) were purchased from Amersharn Buchler (Braunschweig, Germany). For the thinlayer chromatography, silica gel G 60 plates with fluorescent indicators and concentration zone were purchased from E. Merck, Darmstadt, Germany.

The thin-layer radiochromatography analyses were performed with the linear analyzer LB 2870, Berthold Company, Wildbad, Germany.

RBL-l cells (rat basophilic leukemia cells) were donated by Prof. P. Piper, Royal College of Surgeons, London. The medium for RBL-1 cells consisted of Eagles Medium, newborn calf serum,

and fetal calf serum, L-glutamin, and a mixture of penicillin and streptomycin, and was purchased from Gibco, Karlsruhe, Germany. The cells were grown in spinner flasks (Bellco Glass Inc, Vineland, New Jersey, USA).

Lyophilized seminal vesicle microsomes were freshly obtained from slaughtered rams Oulius Kind OHG, Grevenbroich, Germany).

The Ca-Ionophor A 23 187 was purchased from Calbiochem, Frankfurt / Main, Germany. Indomethacin was purchased from Merck, Sharp and Dohme, Rahway, NJ (USA), and Naproxen from Syntex, Palo Alto, CA (USA).

All other solutions and reagents not described were either purchased from Boehringer Mannheim, Mannheim, E. Merck, Darmstadt, or Sigma, Miinchen, Germany.

Methods

Measurement of the Prostaglandin Biosynthesis (Cyclo-Oxygenase Activity)

25 µl Iyophilized microsomes from ram seminal vesicle (1.8mg protein/m/d) are suspended in 975µl calcium phosphate buffer (50mmol/l, pH 7.5), and incubated in the presence of test substances together with 20 µmol/l 14C-arachidonic acid (150,000cpm/ml) for 10 minutes at room temperature.

The incubation reaction is stopped with 20 µl acetic acid and is extracted with 2ml of ethyl acetate. The extract is then compressed under N2 and separated on silica gel plates with a concentration zone in a solvent mixture of ether: hexan: acetic acid (50:50:1). This solvent is not suitable to separate the Prostaglandins but rather to quickly separate the non-metabolized arachidonic acid from its cyclo-oxygenase products. If a separation of the formed prostaglandins is desired, a solvent mixture of ether acetate: acetic acid = 99:1 (3 consecutive separations) is recommended.

The radioactivity distribution on the plate is measured thereafter using the TLC linear analyzer (Berthold Company). The radioactivity of the formed cyclo-oxygenase products (starting peak) and the non-metabolized arachidonic acid (front peak) are calculated as a percentage of the total radioactivity. Measurements are performed in triplicates and the means and standard deviations of the radioactive cyclo-oxygenase products are plotted against the logarithm of the test substance concentration. The concentration of test substance which leads after graphical interpolation to a 50% inhibition of the radioactive cyclo-oxygenase products is noted as IC50-value. Naproxen is used as a positive control of inhibition and is measured in each experiment to determine the IC50 value. The responding volume of the solvent for the test substances is used as blank (20 µl ethanol).

Measurement of Leukotrien Biosynthesis (5-Lipoxygenase Activity)

To search for inhibitors of the leukotrien biosynthesis, cell cultures of RBL-1 cells (rat basophilic leukemia cells) are particularly well suited.

RBL-1 cells are centrifuged for 20 minutes at 400 x g and are adjusted with potassium phosphate buffer (50mmol/ l; pH 7.4) to a cell count of 1.5 x 10 cells/ ml.

Indometacin (10µmol/l), the tested substance in various concentrations, 14C-arachidonic acid (20µmol/l cold plus approximately 100,000cpm radioactive arachidonic acid with a specific radioactivity of 56 mCi / mmol) and the Ca-Ionophor A 23 187 (20 µmol/l) are added to 1 ml of this cell suspension. After an incubation time of five minutes the assay is acidified with 20 µl of acetic acid and thereafter extracted twice with ethyl acetate. The extract is compressed under N2, then again resuspended with 20 µl ethyl acetate, and placed on silica gel thin-layer chromatography plates. The separation of the radioactive reaction products follows with two different solvents at 4 *C.

In the first solvent (ether: hexan: acetic acid=50:50:1) the plates are developed twice in immediate succession. In the second solvent (ethyl acetat:iso-octan: H20: acetic acid=110: 50:10:20; upper phase) the plates are only developed to approximately half the height of the plate. The radioactivity distribution is measured with the Berthold linear analyzer. 5-HETE and the LTB4-1somers (with a

common peak) are separated by these two solvents from arachidonic acid, other monoHETEs (12-HETE and 15-HETE), and phospholipids and triglycerides. The 5-HETE peak and LTB4 peak (in the mixture of isomers of various LTB4 Isomers) are integrated with a TLC-Linear analyzer (Berthold Company) and are expressed as a percentage of the total radioactivity. Measurements are done in triplicate and means as well as standard deviations are plotted on semi-logarithmic paper against the inhibitor concentration. The IC50 value is graphically calculated by interpolation. In each experiment the IC50value for nordihydroguaiaretic acid (NDGA) is measured as a positive control. An equal volume of the used solvent for the test substances is used as blank.

Lyophilized microsomes from ram seminal vesicles are prepared according to the method of van der Ouderraa et al. (16). RBL-1 cells are grown in spinner flasks according to the instructions by Isersky et al. (12). The protein concentrations are measured according to the Lowry et al. method (13).

Results

The effect of both the fat-soluble (fPE) and water-soluble (wPE) pollen extract fractions on the biosynthesis of prostaglandins from radioactively marked arachidonic acid catalyzed by the cyclo-oxygenase in ram seminal vesicle microsomes is shown in Fig. 1.

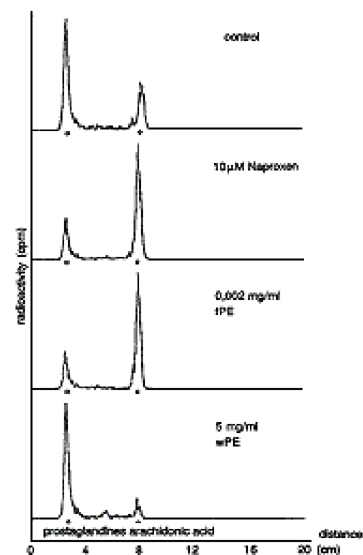

Fig. 1 Effect of the fat-soluble (fPE) and watersoluble (wPE) pollen extract fractions on the prostaglandin biosynthesis from radioactive-marked arachidonic acid in ram seminal vesicle microsomes in comparison to a non-steroidal anti-inflammatory agent (Naproxen).

Under identical conditions 14C-marked arachidonic acid was incubated in the presence of 5mg/ml water-soluble pollen extract (wPE, lowest chromatogram), 20µm/ ml fat-soluble pollen extract (fPE, second radio-chromatogram from bottom), 10 µmol / l Naproxen (non-steroidal anti-inflammatory agent and cyclo-oxygenase inhibitor), as well as 20 µl ethanol (solvent of the utilized test substances) and were incubated with ram seminal vesicles microsomes as the source for the enzyme.

After extraction of the radioactive reaction products and the subsequent thin-layer chromatography separation, two radioactive peaks are obtained. The starting peak contains the different cyclo-oxygenase products (Prostaglandin E2, F2, D2, G2, H2), which are not further separated with the chosen solvent. The front peak contains the rest of the non-metabolized arachidonic acid. A correlative comparison of the four radio-chromatograms shows that the fat-soluble pollen extract in a concentration of 20 µg/ml inhibits the biosynthesis of prostaglandins from arachidonic acid to approximately the same extent as the non-steroidal anti-inflammatory agent and cyclo-oxygenase inhibitor Naproxen in a concentration of 10 µmol/l.

The water-soluble pollen extract shows no significant inhibition of prostaglandin biosynthesis up to a contraction of 5 mg/ml in comparison to the control.

In a similar manner, the concentration-dependent inhibition of prostaglandin biosynthesis by the fat-soluble pollen extract was measured (Fig. 2). Graphical interpolation resulted in an estimated 50 % inhibition of prostaglandin biosynthesis from arachidonic acid by the fat-soluble pollen extract at a concentration of only 5 µg/ml.

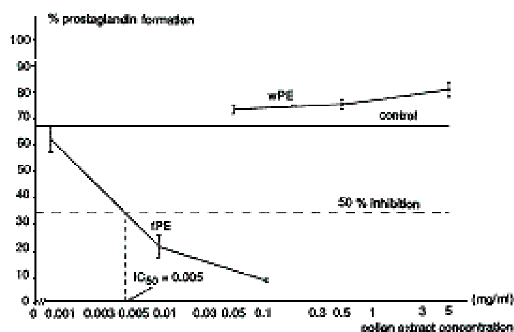

Fig. 2 Determination of the IC50-value for inhibition of prostaglandin biosynthesis (cyclo-oxygenase activity) from arachidonic acid (x ± SD) in ram seminal vesicle microsomes by the fat-soluble (fPE) pollen extract fraction (n = 3, related to the pollen extract concentration). 100% prostaglandin formation corresponds to a complete metabolism of 20µmol/ 1 14C-arachidonic acid.

In a similar fashion, the effect of both pollen extract fractions on the biosynthesis of leukotriens from arachidonic acid was investigated. We utilized cell cultures from rat basophilic leukemia cells (RBL-1 cells) as the source for the enzyme 5-lipoxygenase, which catalyzes the biosynthesis of leukotriens from arachidonic acid.

The effect of both pollen extract fractions on the leukotrien biosynthesis is initially again shown in the thin-layer radiochromatography (Fig. 3).

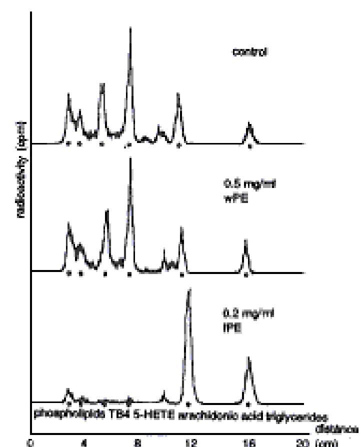

Fig. 3 Effect of the fat-soluble (fPE) and watersoluble (wPE) pollen extract fractions on the leukotrien biosynthesis from radioactive-marked arachiclonic acid in rat basophilic leukemia cells (RBL-1 cells).

Under identical conditions RBL-1 cells were incubated in the presence of water-soluble (0.5 mg / ml) and fat soluble (0.2mg/ml) pollen extract together with the Ca-Ionophor A 23 187 and radioactive arachidonic acid. The three radio-chromatograms shown in Fig. 3 result after extraction of the radioactive reaction products and thin-layer chromatography separation. In the presence of fatsoluble pollen extract (fPE, bottom chromatogram) the enzymatic activity of 5-lipoxygenase is practically completely inhibited. The water-soluble pollen extract, however, shows no significant inhibition of the 5-lipoxygenase reaction (formation of 5-HETE and leukotrien B4-Isomers) in comparison to the control even if a 2.5-fold higher concentration (0.5 mg / ml) is utilized.

A 50 % inhibition of the leukotrien biosynthesis (5-lipoxygenase activity) is reached under these experimental conditions at a concentration of 0.08 mg / ml fat-soluble pollen extract (see Fig. 4). With the watersoluble pollen extract the leukotrien biosynthesis could not be inhibited in concentrations up to 5 mg / ml (data not shown). To judge the inhibitor effect of both pollen extract fractions on the prostaglandin and leukotrien biosynthesis in a therapeutic manner, the IC50-values for some known steroidal and non-steroidal anti-inflammatory agents were measured under the same conditions. Since the concentration of both pollen extract fractions cannot be expressed as a molar concentration, the concentration of the tested anti-inflammatory agents were converted from molarity to mg / ml to allow a better comparison of in vitro effectiveness. In Table 1 the IC50-values for the inhibition of leukotrien and prostaglandin biosynthesis are summarized. Table 1 demonstrates that the fat-soluble pollen extract fraction expressed as mg / ml

inhibits the prostaglandin and leukotrien biosynthesis in vitro more than acetyl salicylic acid does, and equally as strongly as the non-steroidal anti-inflammatory agent diclofenac.

Discussion

The goal of this study was to test the effect of a defined pollen extract on the prostaglandin and leukotrien biosynthesis in vitro to develop a pharmacodynamically plausible hypothesis for its clinical effectiveness in patients with chronic prostatitis, BPH, and prostatodynia (also called prostate congestion [231]).

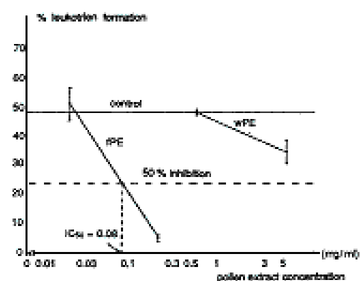

Fig. 4 Determination of the IC50-value for the inhibition of leukotrien biosynthesis (5-lipoxygenase activity) from arachidonic acid (-x ± SD) in rat basophilic leukemia cells (RBL-1 cells) by the fat-soluble (fPE) pollen extract fraction (n = 3, related to the pollen extract concentration). 100 % leukotrien formation corresponds to a complete metabolism of 20µmol/ 114 C-arachiclonic acid.

To determine prostaglandin and leukotrien biosynthesis, thin-layer chromatography was utilized and the radioactivity distribution of the formed cyclo-oxygenase and 5-lipoxygenase products as well as the non-metabolized arachidonic acid was investigated in ram seminal vesicles microsomes and RBL-1-cells.

The TLC documentation of the cyclo-oxygenase activity in this screening method is reliable and complete if influences on the metabolism of cyclo-oxygenase are tested. Among the chemical-analytical methods of measurements for the influence on the 5-lipoxygenase pathway of the arachidonic acid cascade, thin-layer radiochromatography considers the sum of 5-HETE and the LTB4 isomers as representative for the 5-lipoxygenase products formed and does not capture peptidol leukotriens. This method is valuable in screening for 5-lipoxygenase in-hibitors if intact cultivated RBL-1 cells are utilized. To avoid the undesirable metabolism of arachidonic acid by the cyclo-oxygenase, indometacin is utilized in a sufficient inhibitory concentration. This does not affect the 5-lipoxygenase pathway.

The results document an inhibitory effect on the prostaglandin and leukotrien biosynthesis in vitro by the fat-soluble pollen extract. The inhibition of cyclo-oxygenase predominates. The inhibition of both cyclooxygenase and 5-lipoxygenase is dose dependent and the graphically determined IC50-values are approximately equal to those of diclofenac. The water-soluble pollen extract fraction, however, did not show a significant inhibitory effect on the arachidonic acid cascade in vitro.

Tab. 1 Effect of the fat-soluble (fPE) pollen extract fraction on the prostaglandin and leukotrien biosynthesis in direct comparison with other anti-inflammatory agents.

Test substance	IC50-value 5-lipoxygenase (µmol/1)	(mg/ml)*	IC50-value Cclo-oxygenase (µmol/1)	(mg/ml)*
Pollen extract	-	0.08	-	0.005
Naproxen	215	0.0495	8	0.0018
Diclofenac	220	0.0623	26	.00074
Indometacin	240	0.0859	0.35	0.0002
Acetyl salicylic acid	>500	>0.090	375	0.0675
Paramethasone	>100	>0.053	>500	>0.267

* For better comparison of the inhibitory effects, the IC50-values of the anti-inflammatory agents were also expressed in mg/ml.

Clinically the pollen extract has resulted in a reduction of pathologically increased white cell counts in prostatic secretions in patients with chronic non-bacterial prostatitis, with a concomitant decrease in dysuria and discomfort or pain in the inguinal, perineal, or genital area (7). In BPH and concomitant prostatic congestion, which also exhibits histological evidence for chronic inflammation and interstitial

edema and the congestion of secretions in prostatic tissues (9,23), the symptomatic effect of the pollen extract leads to an improvement in the voiding dysfunction (4,5,7,8).

If the chronic inflammatory or congestive changes found in these benign prostatic conditions are considered as the pathophysiologically relevant substrate of the subjective complaints (5,23), the therapeutic effectiveness of the pollen extract could be the result of an intraprostatic inhibition of both the prostaglandin and leukotrien biosynthesis and a subsequent anti-edematous and antileukotactic effect of the fat-soluble fraction according to our in vitro results.

Furthermore, other effects, not primarily related to inflammation, are possibly relevant for the therapeutic mechanism of the pollen extract. The prostaglandin-modulated contraction of smooth muscle cells (19) resulting in coordinated voiding by bladder and urethral smooth muscle might also be influenced by an inhibition of the cyclo-oxygenase. Therefore a relaxation of the prostatic urethra could also explain urodynamic improvements after treatment with pollen extract such as the reduction in residual urine and the improvement in average and peak urinary flow rate (4,5,7,8) (these parameters are found to be abnormal in patients with benign prostatic diseases (6,14,18)). Concerning the 5-lipoxygenase inhibition, no indications for a relaxation of the SRS-A (slow reacting substance of anaphylaxis)-induced contraction in vivo are available.

A further possible pharmacological effect of the pollen extract in patients with BPH could be a prophylactic or pathophysiologically relevant effect concerning hormonal or immunological metabolic processes in the prostate. Prostaglandins and leukotriens are suspected of being involved in the etiology and pathogenesis of BPH as a result of eicosanoid-dependent dysregulations (1, 17). A dose-dependent inhibition of the 5(a-reductase and the 3(a- and 3ß-hydroxysteroid-dehydrogenase which regulate the intraprostatic testosterone metabolism in the epithelium and stroma of BPH homogenates has been documented in vitro for the fatsoluble pollen extract fraction (M. Krieg, personal communication, publication in preparation). Whether and to what extent these results may be connected to our findings and to what extent these results are of pharmacological importance in humans has to be tested in further studies.

Treatment with ß-sitosterin, a phytosterol. also contained in rye pollen (21), has led to a decrease in the prostaglandin concentration in BPH tissue (24) and in the prostatic secretion of BPH patients (2). Concerning the discussed pharmacodynamical effects of the fatsoluble pollen extract fraction with a ß-sitosterin content of 8.3 % (w/w), these in vivo results do not allow any further conclusions since data concerning the above-mentioned metabolic parameters were not measured. The documentation of inhibition of the phospholipase A2 by free fatty acids (3), which are also contained in the fat-soluble pollen extract fraction (30 %; w/w) merely demonstrates that a pharmacological effect on the production of arachidonic acid from phosphatides with subsequently reduced substrate for cyclo-oxygenase and 5-lipoxygenase is possible.

Concerning the use of non-steroidal antiinflammatory agents for benign prostatic diseases, not much is known with the exception of an unsuccessful treatment of non-bacterial prostatitis with ibuprofen (400 mg po tid over 90 days) in a pilot study (22). Clinical experiences with the pollen extract in other typical indications for non-steroidal anti-inflammatory agents are also lacking. A comparison of desirable effects on the basis of in vivo studies is therefore not possible. Side effects associated with a generalized prostaglandin deficiency such as damage to the gastric mucosa (15), as it is characteristic for cyclo-oxygenase inhibition (11), has not been reported after the use of pollen extracts in humans. The side effects known to occur in humans after the use of non-steroidal anti-inflammatory agents are therefore not seen in the treatment with the pollen extract. Gastrointestinal complaints can occur (4, 5), however, but their incidence is rather rare and the intensity of these side effects is mild or moderate.

In drug extracts a number of different chemical compounds are contained some of which, in the case of the pollen extract, ß-sitosterin and free fatty acids, are pharmacologically effective. Therefore a clear determination of the clinical relevant substance or substances and their bioavailability is often not possible. This is particularly true for the pollen extract since even the water-soluble fraction has shown a significant growth inhibition of cultivated prostate cells in experimental studies (F K. Habib, Edinburgh, personal communication, publication in preparation). The possible explanations for the different side effect profiles of pollen extract and non-steroidal anti-inflammatory agents are therefore limited.

If identical conditions are assumed, the clinically utilized daily dosages of pollen extract (fat-soluble fraction) and diclofenac are 12 and 50 mg, respectively, which inhibit in vitro the prostaglandin and leukotrien biosynthesis in an equivalent fashion. Considering in addition the reduction of the production of arachidonic acid by the free fatty acid of the pollen extract, and the inverse relationship between orally taken dose and relative serum concentration as has been demonstrated for diclofenac (20), it is evident that the pharmacologically necessary dose of the pollen extract is comparatively low.

If one assumes a mechanism of action for the fat-soluble pollen extract fraction that is not completely or partially independent in relation to the eicosanoids, it seems reasonable to assume that the pollen extract in the usual dosage does not inhibit local prostagiandin biosynthesis in the mucosal cell layer of the gastrointestinal tract to an extent that it would cause undesirable side effects. At the same time, however, in the prostate and/ or periurethrally, a therapeutically necessary concentration may be reached. The chronic form of congestive and inflammatory processes in benign prostatic conditions which can be treated with a lower concentration of drugs in comparison to the acute inflammatory processes is another indicator for this hypothesis.

Our in vitro experiments concerning the influence of a pollen extract on the arachidonic acid cascade require animal experiments and pharmacological confirmation in humans to determine the value of the assumed therapeutic mechanisms of action, namely anti-congestive, anti-inflammatory, relaxant, and antiproliferative. This does not affect the possible relevance of the watersoluble pollen extract fraction for clinical effectiveness.

In summary we conclude that the in vitro inhibition of the prostaglandin and leukotrien biosynthesis by the fat-soluble pollen extract fraction offers a pharmacologically plausible explanation for the clinical effectiveness and the underlying mechanism in the therapy of benign prostatic conditions with the pollen extract.

Summary

A standardized extract mainly from rye pollen (Cemilton®) was tested in vitro on the inhibition of prostaglandin and leukotrien synthesis. The determination of the prostaglandin and leukotrien synthesis from labelled arachidonic acid was done in microsomes of ram seminal vesicles and in rat basophilic leukemia cells (RBL-1 cells). The water-soluble and fat-soluble extract fraction from the whole pollen extract were tested separately. The radio-TLC separation of the reaction metabolites showed a dose-dependent inhibition of the cyclo-oxygenase and the 5-lipoxygenase activity by the fat-soluble pollen extract fraction. The IC50-values of 0.005 mg/ml and 0.08 mg/ml, respectively, were similar to those of diclofenac, which was also tested. The water-soluble fraction showed no effect on this test system. According to these in vitro results and clinical experience with the pollen extract so far, its therapeutic efficacy on benign prostate diseases is best explained by the anticongestive, anti-inflammatory effect of the fat-soluble fraction. Due to the different actions of prostaglandins and leukotriens, relaxant and antiproliferative effects are also conceivable.

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[1] Cernilton®; composition: 23 mg pollen extract consisting of 20 mg water-soluble and 3 mg fat-soluble extract fractions. Pharma Stroschein (licensed by Cernitin ™ SA, Lugano, Switzerland), Hamburg.

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