

LIVER SUPPORT:

GRAMINEX Flower Pollen Extract

The Effect of the Pollen Extracts Quercitin and Cernitin on the Liver, Lungs, and Stomach of Rats Intoxicated with Ammonium Fluoride

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Quercitin and Cernitin are not in themselves toxic to rats. When administered at the time of intoxication of the animals with ammonium fluoride, they reduced the noxious effects of the toxic agent on the liver and lungs. It is suggested that Quercitin and Cernitin might play a protective role during prolonged exposure to ammonium fluoride. Neither ammonium fluoride nor Quercitin or Cernitin seem to exert any effect on the stomach.

Key words: pollen extracts, Quercitin, Cernitin, liver, lungs, stomach, ammonium fluoride.

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Fluoride compounds are one of the most potent ecotoxins (MARIER 1972; GROTH 1975; MARKIEWICZ 1981). Several publications discuss the toxic action of fluorine and the problem of reducing its emission (GUMINSKA 1981; MARKIEWICZ 198; DOMINICZAK *et al.* 1982; HUMICZEWSKA *et al.* 1989). In the search for cheap, easily available pharmacological means, devoid of side effects, which would reduce the harmful changes occurring with prolonged intoxication with fluorides, attention was paid to pollen extracts. The latter, which have been employed for years in phytotherapy (c.f. OZAROWSKI 1982), were found to be very useful in various diseases of the kidneys and liver, playing a role also in detoxication processes (SCHWARTZ *et al.* 1982; KULAWIAK 1986). New, previously not described, characteristics of pollen were revealed in investigations carried out in the Department of Pharmacology and Toxicology of the Pomeranian Medical Academy in Szczecin (KULAWIAK 1986; CEGLECKA 1991, 1991a; MYSLIWIEC 1992). However, those studies do not cover all the possibilities of exploiting pollen extracts. Further investigations are therefore needed to establish their pharmacological characteristics and, possibly, other appliances.

The aim of the present study was to investigate the histological and histochemical changes occurring in the lungs, liver, and stomach of rats exposed to ammonium fluoride (NH₄F), and to assess the possible beneficial effect on such changes of two pollen extracts, Quercitin and Cernitin, known as detoxicating agents.

Materials and Methods

Animals

The investigations were carried out on 160 inbred, male Wistar rats, weighing approximately 300g each. Throughout the experiment the animals were fed a standard granulated chow and received water *ad libitum*.

Exposition to ammonium fluoride (NH₄F)

The animals were placed in a toxicological chamber in which the parameters of humidity and temperature were adapted each time to those prevailing in the animal room. The air-flow through the chamber was 10m³/h. Ammonium fluoride was introduced as

aerosol at a concentration of 2mg/m³ of air, and controlled constantly by means of an iono-selective fluoride electrode. The above concentration corresponds to the so-called Highest Permissible Concentration established for men exposed to fluoride compound at 0,0016mg/m³ of air (Decree of the Polish Council of Minister, September 30, 1980).

In the present investigation the animals were exposed to ammonium fluoride for 6 h daily, 5 days a week.

Pollen extracts

As pollen extracts Quercitin and Cernitin were applied. Quercitin (synthesized in the Department of Inorganic Chemistry of the Rzeszow Branch of the Krakow Technical University) is a mixture of sodium

salts of quercetin 8.5 disulphonic acid i.e of Na₂QDSA in which NaQSA-5' and NaQSA-8 appear at the ratio 1:1 (unpublished data).

Cernitin (AB Cernelle, Vegeholm, Sweden) appears in two forms, as a fraction soluble in water (Cernitin T60), and as a fraction soluble in lipids (Cernitin GBX). Cernitin T60 contains from 60 to 92% of aminoacids, and Cernitin GBX from 10 to 16% of phytosterols (NIELSON *et al.* 1987, SEPPANEN 1989). In medicine mixtures of the two fractions are used (NIELSON *et al.* 1987).

On days when the rats were exposed to ammonium fluoride the appropriate groups also received Quercitin and Cernitin preparations, previously added to their chow. The doses applied are given below.

Grouping of animals

The animals were divided into two series, each comprising 8 groups of 10 rats.

Those of series I (Groups 2-8) were exposed to ammonium fluoride and/or given pollen-extracts for 3 months, while those of Series II (Groups 10-16) underwent the same experimental procedure but for 6 months.

Group 1 was the control for Series I, and Group 9 for Series II. The two control groups were neither exposed to NH₄F, nor given pollen extracts and remained throughout the experiment in the animal room.

Groups 2 and 10 received Quercitin at dose I, i.e. 30mg/kg b.w./day.

Groups 3 and 11 received Quercitin at dose II, i.e. 20mg/kg b.w./day.

Groups 4 and 12 received Cernitin T60 (100 mg/kg b.w./day and simultaneously, Cernitin GBX (200mg/kg b.w./day).

Groups 5 and 13 were exposed to NH₄F only.

Groups 6 and 14 were exposed to NH₄F and received Quercitin at dose I (as the animals of Groups 2 and 10, respectively).

Groups 7 and 15 were exposed to NH₄F and received Quercitin at dose II (as the animals of Groups 3 and 11, respectively).

Groups 8 and 16 were exposed to NH₄F and received Cernitin (as the animals of Groups 4 and 12, respectively).

After conclusion of the experiments (Series I, i.e. Groups 2-8, and Control Group 1 after 3 months), and Series II, i.e. Groups 10-16, and control Group 9 after 6 months the animals were killed by decapitation.

Histology and histochemistry

After killing the animals, the lungs, liver, and stomach were dissected out. Tissue specimens intended for histological examination were fixed in Bouin's fluid, embedded in paraffin, sectioned at 8µm, and stained with Mayer's haematoxylin and aqueous eosin.

For histochemical analysis the tissues were immediately frozen, and after cut on a cryostat into 10µm sections. Histochemical reactions performed on this (unfixed) material included (1) succinic dehydrogenase (SDH) using sodium succinate as substrate, according to Nichlas (PEARSE 1968), (2) acid phosphatase (AcP), and (3) alkaline phosphatase (AIP), using sodium-glycerophosphate as substrate according to Gomori, (PEARSE 1972). Following incubation at 37° C (SDH for 30min AcP and AIP for 60min), the sections were embedded in glycerol-gel. In order to confirm the specificity of the particular enzymatic reactions, control reactions without the substrates were simultaneously run.

Results

Histological observations

Haematoxylin and eosin (HE) stained sections of the liver, lung, and stomach of control animals (Groups 1 & 9) showed that the morphological picture of all the investigated organs was normal (Figs 1&5).

L i v e r. In the liver of experimental animals exposed to ammonium fluoride for 3 months (Group 5) the liver cells appeared brighter, this being caused by excessive accumulation of glycogen. The blood vessels were extended, and in places fibrosis could be seen (Fig. 2).

After 6 months exposure to NH₄F (Group 13), apart from the changes described above, the laminar structure of lobules was obliterated, particularly at their peripheral parts. Liver cells seemed to be diffused, and no clear borders between them were seen. The connective tissue strands were more extensive (Fig. 3).

In the animals of groups 2, 3, and 4, which for 3 months received pollen-extracts only, no differences in comparison with control Groups 1 and 9 were detected. Similarly, no changes were found in the liver of animals given only pollen extracts for 6 months (Groups 10, 11 & 12).

Rats exposed for 3 months to ammonium fluoride, but receiving simultaneously Quercetin at dose I or II (Groups 6&7), of Cernitin (Group *) also did not reveal any differences., compared with controls.

The same was true for animals of Groups 15 and 16 (intoxicated for 6 months, but simultaneously given Quercetin at dose II or Cernitin). However, in the rats of Group 14 (exposed to NH₄F for 6 months, and given Quercetin at dose I), some parts of the liver showed obliteration of the interlobular blood vessels (Fig. 4).

L u n g s. The results of histological observation of the lungs are summarized in Table 1.

No pathomorphological changes were visible either in control Groups 1 and 9, or in rats which received pollen extracts only (i.e. Groups 2, 3, 4, 10, 11 & 12).

Fig. 1. Liver of a control rat (Group 9); identical pictures were observed in control Group 1. Hex 140.

Fig.2. Liver of a rat exposed for 3 months to ammonium fluoride (Group 5). Note the brightening of liver cells, the extension of blood vessels, and signs of fibrosis Hex 150. Fig.3. Liver of a rat exposed for 6 months to ammonium fluoride (Group 13). Note the extension of connective tissue strands Hex 150. Fig. 4, Liver of a rat exposed for 6 months to ammonium fluoride, but simultaneously receiving Quercetin at dose I (5mg/kg/day), (Group 14). Note some extension of capillaries, and, in at places, obliteration of the laminar structure HE x 150. Fig.5. Lung of control rat (Group 9); identical pictures were observed in control Group 1. HE x 150. Fig.6. Lung of a rat exposed for 6 months to ammonium fluoride (Group 13). Note numerous extravasations of erythrocytes HE x 150.

Fig.7. Lung of a rat exposed for 6 months to ammonium fluoride (Group 13). Note general oedema and, in the alveoli, effusional fluid. Erythrocytes, macrophages and desquamated respiratory epithelial cells HE x 150.

Fig.8. Lung of a rat exposed for 6 months to ammonium fluoride (Group 13). Note concretions of haemosiderin in the macrophages HE x 150. Fig.9. Lung of a rat exposed for 6 months to ammonium fluoride (Group 13). Note numerous accumulations of acidophilic leucocytes HE x 150. Fig., 10. Lung of a rat exposed for 6 months to ammonium fluoride (Group 13). Note numerous accumulations of limphoidal cells HE x 150. Fig.11. Lung of a rat exposed for 3 months to ammonium fluoride (Group 5). Note the extension of alveoli filled with effusional fluid HE x 150. Fig.12. Liver of a control rat (Group 1). The activity of succinic dehydrogenase is moderate x 150. Fig.13. Liver of a rat exposed for 6 months to ammonium fluoride

(Group 13). The activity of succinic dehydrogenase is weak x 150. Fig.14. Lung of a control rat (Group 1). The activity of succinic dehydrogenase is moderate x 150. Fig. 15. Lung of a rat exposed for 3 months to ammonium fluoride (Group 5). The activity of succinic dehydrogenase is weak x 150.

In the remaining experimental groups, which were all exposed to ammonium fluoride, more or less frequent or pronounced extravasations of erythrocytes, lymphoedema, and hypertrophy of the lymphatics could be observed. These symptoms were strongest in animals intoxicated only with ammonium fluoride for 6 months (Group 13), in which in the alveoli not only effusional fluid but also desquamated respiratory epithelium cells (Fig.7), erythrocytes, and macrophages were visible. The latter could be seen also in the interalveolar septa, and within the respiratory epithelium (Fig. 6). In the macrophages numerous large concretions of haemosiderin (Fig.8), originating as the result of phagocytosis of erythrocytes, could be seen. In Group 13 also very distinct hypertrophy of the lymphatics was visible. This was particularly evident around the blood vessels and bronchioli, where often infiltrations of lymphoidal cells (Fig.9) were observed.

Similar changes, but less intense, were found in Groups 14, 15 & 16 (NH₄F intoxication for 6 months, and simultaneous applications of Quercetin or Cernitin), and in Group 5 (rats exposed only to NH₄F for 3 months), (Fig.11). In Groups 6, 7&8 (intoxication for 3 months plus simultaneous application of Quercetin or Cernitin), the morphological picture of the lungs did not essentially differ from that in controls, the only difference being the accumulation of small quantities of effusion in some of the alveoli.

S t o m a c h. In the stomach only the morphological characteristics of the mucous membrane were analysed. Neither in the surface and glandular epithelium, nor in the lamina propria and muscularis mucosae could any differences between experimental and control animals be observed.

Histochemical observations

The results of histochemical observations are summarized in Table 2.

Succinic dehydrogenase (SDH)

L i v e r. In control animals (Groups 1&9) succinic dehydrogenase appeared as a microgranular reaction, which was usually strongest around the central vein of the lobules. General, SDH activity in both control groups could be classified as moderate (Fig. 12).

Table 1 Morphological changes in the lungs of rats exposed to ammonium fluoride (NH₄F) and/or to the

pollen extracts Quercetin (Qu.I, dose 5mg/kg. B.w./day; Qu.II dose 20mg/kg b.w./day) or Cernitin (C., dose 200mg/kg b.w./day) during 3 and 6 months

Table 2 Activity of succinic dehydrogenase (SDH), acid Phosphatase (AcP), and alkaline phosphatase (AIP) in the liver lungs, and stomach of rats exposed to ammonium fluoride (NH₄F), and/or to the pollen extracts Quercetin (Qu. I, dose 5mg/kg b.w./day; Qu. II, dose 20mg/kg b.w./day), or Cernitin (C., dose 200mg/kg b.w./day) during 3 and 6 months

In rats exposed only to ammonium fluoride for 3 or 6 months (Groups 5&13) SDH activity decreased (Fig. 13).

In the remaining groups, i.e. in both those exposed to NH₄F and receiving pollen extracts only, the activity of SDH did not differ from that observed in the controls.

L u n g s. In untreated control rats moderate SDH activity was observed in all cells of the interalveolar septa and in the walls of bronchioles. In the ciliated epithelium of the latter and in the blood vessels it was higher and could be classified as strong (Fig. 14).

In Groups 5 and 13 (exposed to NH₄F for 3 and 6 months, respectively), and in Groups 2 and 10 (animals intoxicated with ammonium fluoride, and given Quercetin at dose I during 3 and 6 months, respectively), the reaction was less intense (Fig. 15), the only exception being alveolar phagocytes in which SDH activity was in all the mentioned groups fairly strong.

In Groups 3 and 11 (Quercetin at dose II for 3 and 6 months, respectively), and in Group 12 (Cernitin for 6 months) the activity of succinic dehydrogenase was higher than that in the controls, particularly within the endothelium, ciliated epithelium, and in the phagocytes present in the lumen of alveoli and bronchioles (Fig. 16). In all the remaining groups, the reactions for SDH were comparable to those described in animals of the untreated control groups.

S t o m a c h. In the stomach of the control animals (groups 1 & 9) very strong SDH activity was observed in the glandular epithelium, in the lamina propria it was moderate, while in the surface epithelium and muscularis mucosae it remained rather weak.

In none the experimental groups, i.e. intoxicated and/or treated with pollen extracts, did the activity of SDH differ from that found in the controls.

Acid phosphatase (AcP)

L i v e r. In both control groups (Group 1 and 9), the reaction for AcP was in all cells of the liver, including

Kupffer cells (Fig. 17), fairly strong. Intoxication with NH₄F for 3 or 6 months (Groups 5 and 13) reduced AcP-activity in liver cells to weak, and in Kupffer cells (Fig. 18) to moderate. In all the other groups, exposed to NH₄F and/or treated with pollen extracts, the activity of AcP in the liver did not differ essentially from that in controls.

L u n g s. In control animals (Groups 1 & 9), and in those receiving pollen-extracts only (Groups 2, 3, 4, 10, 11 & 12) the cells of the interalveolar walls, as well as the epithelial cells of alveoli, bronchi, and bronchioli, revealed moderate AcP activity, the reaction being stronger only in the granular pneumocytes (Fig. 19). Intoxication for 6 months with NH₄F, with or without simultaneous treatment with pollen extracts (Group 12, 14, 15 & 16), brought about a distinct increase in the activity of AcP, which was particularly evident in the granular pneumocytes and in other cells of the interalveolar walls (Fig. 20).

S t o m a c h. In control Groups 1 and 9 the reaction for AcP was in the glandular epithelium strong, in the surface epithelium and the muscularis mucosae moderate, and in the lamina propria weak. Intoxication with NH₄F and/or treatment with pollen extracts did not cause in any of the experimental groups changes in the above-described situation.

Alkaline phosphatase (AIP)

L i v e r. The reaction for AIP in the lungs of control animals (Group 1 & 9) was very weak (Fig. 21). In groups exposed to ammonium fluoride only (Groups 5 & 13) AIP activity increased to moderate (Fig. 22), while in all the other ones it did not differ from that in the controls.

L u n g s. AIP activity in the lungs of control Groups 1 and 9 was fairly evenly distributed in the interalveolar walls, and generally moderate, a slightly more intense reaction being observed in the endothelium of bronchioli and in alveolar pneumocytes (Fig. 23). In animals intoxicated with ammonium fluoride only (Groups 5 & 13), the activity of AIP was very strong, while in those receiving Quercetin, both at dose I and II, and not exposed to NH₄F (Groups 2, 3, 10 & 11), it was moderate (Fig. 24). In all the other groups (Groups 6, 7, 8, 14, 15 & 16) the activity of AIP was comparable to that in controls.

S t o m a c h. AIP activity was found mainly in the surface and glandular epithelium. It was similar in all the experimental and control groups, and could be classified as moderate.

Discussion

Earlier studies (DOMINICZAK & SAMACHOWIEC 1982; HUMICZEWSKA *et al.* 1989), as well as the

present one, showed that ammonium fluoride causes various pathological changes in the liver and lungs of rats. It is possible, however, that these changes are not only local responses of the investigated organs but also reflect more general reactions of the whole organism. In the case of the liver it should be borne in mind that it normally accumulates substantial amounts of toxic substances and therefore that, any damage to it may have further, far-reaching consequences.

Prolonged intoxication with ammonium fluoride brings about obliteration of the laminar structure of liver lobules, and more or less extensive fibrosis. These observations are similar to those described in rats with cirrhosis, which developed following intoxication with carbon tetrachloride (GEORGIJEV & KALCZAK *et al.* 1982), hydrogen fluoride (HUMICZEWSKA *et al.* 1989), and the herbicide Simazin (HUMICZEWSKA *et al.* 1990a).

Although, the changes described in the present investigation were slightly less severe than those described in the papers quoted above, it was interesting to note that when intoxication with ammonium fluoride was accompanied by the simultaneous application of the pollen extracts Quercitin or Cernitin, damage to the liver practically did not occur.

Also affected by fluoride are the lungs. Apart from their known role in various physiological and pathological processes, including the metabolism of many biologically active substances, they also participate in the detoxication of the organism (WATTENBERG & LEONG 1965; HEINEMANN & PISMANN 1969; DOLOFF 1971).

Fig.16. Lung of a rat for 6 months receiving only Quercitin at dose II (20mg/kg b.w./day). (Group 11). The activity of succinic dehydrogenase is strong x 150. Fig.17. Liver of a control rat (Group 9). The activity of acid phosphatase is strong x 150. Fig.18. Liver of a rat exposed for 3 months to ammonium fluoride (Group5). The activity of acid phosphatase is weak x 150. Fig.19. Lung of a control rat (Group1). The activity of acid phosphatase is weak x 150. Fig.20. Lung of a rat exposed for 6 months to ammonium fluoride (Group13). The activity of acid phosphatase is very strong x 150. Fig.21. Liver of a control rat (Group 9). The activity of alkaline phosphatase is very weak x 150. Fig.22. Liver of a rat exposed for 6 months to ammonium fluoride (Group 13). The activity of alkaline phosphatase is moderate x 150. Fig.23. Lung of a control rat (Group 9). The activity of alkaline phosphatase is moderate x 150. Fig.24. Lung of a rat exposed for 6 months to ammonium fluoride (Group 13). The activity of alkaline phosphatase is strong x 150. In the lungs of rats intoxicated with ammonium fluoride, numerous acidophilic leucocytes appear in the interstitial tissue, and an all-over increase in the number of lymphoid

cells takes place. While these reactions seem to be non-specific and reflect the activation of the general defense mechanisms of the organism, the observed simultaneous increase in the number of pneumocytes and the appearance of macrophages are probably the result of defensive processes of the lung itself, aimed directly at the toxic agent.

A similar increase in the number of acidophilic leucocytes, lymphocytes, pneumocytes and macrophages was found in many other pathological states of the lungs (PARAFINIUK *et al.* 1975; HUMICZEWSKA *et al.* 1990). Often it is also accompanied by more or less extensive extravasations of blood cells, indicating damage to the capillary walls. Injuries to the endothelium of capillaries, increasing their permeability and in consequence causing extravasations, were also described following intoxication with other substances (such as, e.g., benzene and phosphorus), during infectious diseases, and in diseases of the haemopoietic tissue (cf. DOLOFF 1971).

Blood cells extravasated into the surrounding tissues are recognized there as foreign bodies, and induce inflammatory reactions, during which they are imbibed by the accumulating phagocytes. As a result, macrophages often contain haemosiderin concretions.

The application of Quercitin or Cernitin to rats which were at the same time intoxicated with ammonium fluoride substantially reduced the pathological processes described above. The frequency of extravasations was much lower, which suggests that the pollen-extracts, which the animals received had a positive effect also on the capillaries, reducing their fragility.

The results of histochemical studies revealed that ammonium fluoride also affects the metabolic processes in the liver and lungs, but apparently not in the stomach.

The decrease in succinic dehydrogenase activity in the liver and lungs suggests that in their cells the citric acid (Krebs) cycle was blocked, which could be the result of a negative effect of F ions on these processes (MACHOY 1981, 1987).

Acid and alkaline phosphatases are similarly considered to be sensitive indicators of disturbances occurring in the course of metabolic processes. The increased activity of AIP in the liver and lungs might in this case reflect the pathological changes described in these organs following NH₄F intoxication. As suggested by SAWICKA (1980), an increase in the level of alkaline phosphatase is often connected with abnormalities in transmembrane transport.

The behavior of acid phosphatase was different, intoxication with ammonium fluoride reducing its

activity in the liver, but increasing it in the lungs. On the basis of *in vitro* experiments, GALKA and OGONSKI (personal communication) reached the conclusion that F ions block the activity of acid phosphatase by binding the Mg +2 ions which are necessary for AcP activation. However, it would be difficult to explain in these terms the increase in AcP activity in the lungs, unless one assumes that either in the organism there are mechanisms which counteract the binding of Mg+2 and F ions or that the quantities of F ions reaching the particular organs are too small to block acid phosphatase. In other histochemical and biochemical studies AcP activity was not affected or was even slightly increased following the introduction of fluoride ions (c.f. MESSER & SINGER 1976).

The investigated pollen extracts Quercitin (at dose I or dose II) and cernitin, when applied evoked practically no negative side effects, but when given to animals simultaneously intoxicated with ammonium fluoride, they substantially reduced its negative action, or even prevented the development of negative changes. This demonstrates that Quercitin and Cernitin should be considered as protective agents in cases when prolonged exposition to fluorides is expected. Unfortunately, so far nothing is known about the mode of action of Quercitin and Cernitin, hence further investigations are needed.

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