Acta vet. scand. 1987, *28*, 313-320

From the Department of Pharmacology and Toxicology and Department of Internal Medicine, Norwegian College of Veterinary Medicine, Oslo, and Norwegian Defence Research Establishment, Division for Toxicology, Kjeller, Norway.

Neurotoxicological Examination of the Piglet Brain after Prenatal and Postnatal Exposure to Trichlorfon

By *Gunnar N. Berge, Frode Fonnum, Nils E. Søli and Erling Søgnen*

Berge, G.N., F. Fonnum, N.E. Søli and E. Søgnen: Neurotoxicological examination of the piglet brain after prenatal and postnatal exposure to trichlorfon. Acta vet. scand. 1987, 28, 313-320. – Piglets were given 10 repeated doses of the organophosphorus compound trichlorfon during the postnatal period in order to examine the effect on the brain development (Experiment 1).
Following prenatal exposure to trichlorfon, the ability of a presumptive hypoplastic cerebellum and cerebrum at birth, to regenerate postnatally was investigated (Experiment 2).
Administration of repeated doses of trichlorfon postnatally was accompanied by only small changes in brain weights, morphology and transmitter enzyme activity (choline acetyltransferase, glutamate decarboxylase, aromatic amino acid decarboxylase) in 35 days old piglets. Animals exposed prenatally, and sacrificed at the age of 35 days, showed a significant increase in brain weights and enzyme activities. The animals did, however, not reach control values in cerebral weight, cerebellar weight or total enzyme activities. Morphological changes still showed regional loss of Purkinje cells in the cerebellum. The study clearly indicated that the pig brain was less vulnerable to trichlorfon in the postnatal period of development than when exposed to the compound prenatally.

organophosphorus compound; CNS toxicity; regeneration.

Introduction

Several reports in the last years suggest that the organophosphorus compound trichlorfon (Neguvon®) produces ataxia, tremor and a pronounced cerebellar hypoplasia in newborn piglets born by sows medicated during certain periods of gestation (*Kronevi* 1977, *Kronevi & Bäckstrøm* 1977, *Kronevi & Lindquist* 1978, *Knox et al.* 1978, *Fatzer et al.* 1981, *Gamlem et al.* 1983). In a recent investigation in pigs, *Berge et al.* (1987) observed that not only the cerebellum, but also the total brain was reduced in weight. The histological findings were characterized by a well preserved cerebellar lamination but with a periodical loss of Purkinje cells. The activities of the transmitter synthesizing enzymes choline acetyltransferase, glutamate decarboxylase and aromatic amino acid decarboxylase in the cerebellum were also found to be affected.

Previous studies and reports have dealt with the toxic effect of trichlorfon exclusively as a result of prenatal administration in the pig. The purpose of these experiments was to study the effect of trichlorfon in the postnatal

period where, according to *Davison & Dobbing* (1966) and *Dickerson & Dobbing* (1967), the growth of the pig brain is at its maximum.

In addition, we wanted to study the development of the pig brain postnatally after prenatal treatments with trichlorfon. The parameters studied were brain weight, morphology and transmitter enzyme activity.

Material and methods

Chemical

The organophosphorus insecticide trichlorfon, dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate, was used as Neguvon® powder produced by Bayer AG, Leverkusen, Federal Republic of Germany. One hundred g of Neguvon® contained 97 g of trichlorfon.

Experimental design

Experiment 1: One litter of 8 newborn piglets of the Norwegian Landrace was used in Experiment 1. The litter was divided into 2 groups with 4 piglets (2 ♂, 2 ♀) in the experimental group and 4 piglets (3 ♂, 1 ♀) in the control group.

The piglets were fed naturally during the first 2 weeks post partum, and then 3 times a day with a commercial Norwegian milk replacer in the subsequent 3 weeks of the experimental period. The litter was housed together until slaughter.

Since there is little information available in the literature concerning toxicity of trichlorfon in the postnatal period of the pig, a preliminary experiment was conducted to determine a suitable dose. Two piglets (A and B) were given a maximally tolerated dose (allowing symptoms like hypersalivation, but not muscular fasciculation) of trichlorfon intraperitoneally every third day in a period extending from 1 day after delivery until day 30 post partum. The average tolerated single dose was 66 mg trichlorfon per kg (range from 50-75 mg/kg) in piglet A and 57 mg trichlorfon per kg (range from 40-90 mg/kg) in piglet B.

The dose of trichlorfon administered to piglets in Experiment 1, was therefore chosen to be 50 mg/kg. The Neguvon®-powder was dissolved in 0.9% NaCl immediately before each administration and given as in the preliminary experiment. The piglets were observed clinically after every administration. Toxic symptoms such as hypersalivation and leg weakness occurred in the piglets in connection with 3 of the 10 medications during the experimental period. The intoxicated animals were in these cases given 0.1 mg atropin/kg s.c. to reverse the clinical symptoms. The dose of trichlorfon was subsequently reduced (i.e. to 35 mg/kg) at the time of the next administration.

Five weeks post partum, i.e. 5 days after the last injection of trichlorfon, the piglets were weighed and sacrificed by CO_2-anesthesia and decapitation. Blood samples were collected into heparinized tubes in order to examine the activity of blood acetylcholinesterase. The brain was separated from the spinal chord immediately behind the obex and weighed. The cerebellum and other brain regions were dissected and weighed. In order to calculate the increase in cerebellum - and total brain weight from term to day 35 p.p, the values in this experiment were compared with the weights obtained from a litter of a non-treated sow examined perinatally (mean weight of the total brain 31.53 g, mean weight of the cerebellum 3.13 g, *Berge et al.* 1987). One half of the cerebellum was immersed in 10% formalin for histological examination. The other half was homogenized in cold 0.32 mol/l sucrose for determination of choline acetyltransferase, glutamate decarboxylase and aromatic amino acid decarboxylase.

The samples for histological examination were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Experiment 2: The aim of this experiment was to investigate if piglets with a presumptive hypoplastic cerebellum at term were able to regenerate during a period of 5 weeks post partum.

One sow was given Neguvon® orally at a dose of 60 mg/kg on day 77 and day 87 of gestation. The Neguvon®-powder was dissolved in 0.5 l of water and given by gavage. Seven of the delivered piglets were selected at random and examined. Cerebellar hypoplasia (mean weight 1.92 g) was confirmed in these piglets as compared to untreated controls (mean weight 3.13 g, *Berge et al.* 1987). The remaining 5 piglets in the litter were subsequently housed, fed and examined as described in Experiment 1.

Enzyme assays

Three neurochemical parameters, which are used as markers for different transmitter systems, were examined. The neurochemical parameters were either compared on wet weight basis (specific activities) or compared to the total activity in the cerebellum (specific activity × cerebellar weight).

Enzymes were assayed by radiochemical procedures. The labelled substrates, (1-^{14}C) acetylcholine, (1-^{14}C) acetylCoA, L-(1-^{14}C)-glutamic acid and DL-3.4-dihydroxy (2-^{14}C) phenylalanine were purchased from The Radiochemical Centre, Amersham, England.

Acetylcholinesterase (AChE) activity was measured according to the method of *Sterri & Fonnum* (1978). Choline acetyltransferase (ChAT) was assayed by the micromethod of *Fonnum* (1975).

Glutamate decarboxylase (GAD) was determined by the method of *Albers & Brady* (1959), as modified by *Fonnum et al.* (1977).

Aromatic amino acid decarboxylase (AAD) was assayed as described by *Broch & Fonnum* (1972).

The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Results

Repeated administration of trichlorfon postnatally resulted in a moderate inhibition of acetylcholinesterase activity in the blood of 35 days old piglets. Five days after the last dose, the average remaining activity was 72%.

No clinical symptoms were present in the piglets at the time of examination.

Body and brain weight

The comparison between postnatal and prenatal effects of trichlorfon administration on the piglet body weights and brain weights is presented in Table 1.

There was no significant effect on the body weights of 35 days old piglets, neither as a consequence of prenatal nor postnatal administration of trichlorfon.

In Experiment 1 the mean weight of the total brain was 50.69 g versus 57.22 g in the controls, which represented a small, but significant reduction (11%).

The weight of the cerebellum in the experimental animals showed a reduction of 9% which was, however, not significant. There were no significant differences in the cerebellum/total brain ratio and cerebellum/body weight ratio between postnatally medicated and non-medicated animals (Table 1).

In the 35 days old control animals the average increases in cerebellum and total brain weights were 105% and 82%, respectively, compared to the weights obtained from a litter of a non-treated sow examined perinatally. Corresponding values were slightly reduced in the medicated animals (87 and 61%, respectively).

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Table 1. Comparison between postnatal and prenatal effects of trichlorfon administration on piglet brain weights (mean ± SD).

		Number of piglets	Age (days)	Body weight of the piglets (g)	Total brain weight (g)	Weight of Cerebellum (g)	Cerebellum/ total brain ratio (%)	Cerebellum body weight ratio (%)	Increase in cerebellum weight from partus	Increase in total brain weight from partus
Experiment 1 (sow A)	Controls	4	35	11875 ±3304	57.22[a] ±1.72	6.42 ±0.53	11.2	0.054	3.29g* (105%)	25.69g* (82%)
	Postnatal medication	4	35	10750 ±1850	50.69[b] ±1.68	5.85 ±0.16	11.5	0.054	2.72g* (87%)	19.16g* (61%)
Experiment 2 (sow B)	Prenatal medication	7	1-3	1479 ±414	25.34 ±1.42	1.92 ±0.20	7.6	0.130	-	-
		5	35	10600 ±2100	41.81 ±1.91	3.91 ±0.52	9.4	0.036	1.99g (104%)	16.47g (65%)

ab: $p < 0.05$

* Weight differences from comparison with data from a litter of a non-treated sow examined perinatally: mean weight of the total brain 31.53 g, mean weight of the cerebellum 3.13 g (*Berge et al.* 1987).

Table 2. Weights of selected regions of the brain in 35 days old piglets following repeated administration of trichlorfon in the postnatal period (mean ± SD)

	Striatum (g)	Hippocampus (g)	Colliculus sup. et inf. (g)	Cortex (g)
Control (n = 4)	1.55 ± 0.18	1.71 ± 0.11	0.87 ± 0.10	34.98 ± 1.67
Medicated (n = 4)	1.26 ± 0.18	1.45 ± 0.13	0.90 ± 0.04	31.56 ± 1.40

The mean weight of the cerebellum in 35 day-old piglets, whose mother had been exposed to trichlorfon during pregnancy (Experiment 2), was much less than in the controls (3.91 g versus 6.42 g). In spite of the great differences in the total weight values at this age, the relative weight increase from term to day 35 was similar for the 2 groups (104% versus 105%). Furthermore, the weight of the total brain was also less in the exposed piglets in this experiment. The average increase in weight from term was 65% versus 82% in the control group. In addition, the weight of the cerebellum as a percentage of total brain weight was calculated to 9.4%, which was less than that of the postnatally medicated animals and controls. The weights of some selected brain regions are presented in Table 2. No significant variations were observed.

Morphological examination of cerebellum

The histology of the cerebellar cortex looked remarkably similar in the controls and the experimental piglets. In Experiment 1 no consistent difference could be found between controls and piglets treated postnatally (Compare Fig. 1 A, B and C with G, H). However, on comparison of the cerebellum from prenatally treated animals (Exp. 2) and controls, some slight differences appeared to be consistent (Fig. 1). Thus, the external granular layer was less well developed and regions with lack of Purkinje cells occurred more frequently in the experimental animal than in the controls (Compare Fig. 1A, B and C with D, E and F).

Neurochemical examination of cerebellum

Following postnatal medication of trichlorfon the transmitter synthesizing enzymes ChAT, GAD and AAD in the cerebellum appeared to be only slightly affected or within normal range (Table 3). The specific activity of the enzymes were relatively similar in experimental and control animals (Experiment 1). The total cerebellar activity of ChAT, GAD and AAD was 92.8%, 86.1% and 80.0% of control values.

In prenatally exposed piglets at 35 days post partum *(Experiment 2),* the specific enzyme activities were significantly higher than in the control animals at the same age *(Experiment 1).* The total activities of ChAT, GAD and AAD were, however, slightly reduced, i.e. to 92.5%, 71.3% and 87.6% of the control values, respectively.

Discussion

Postnatal administration of trichlorfon in piglets was accompanied by only small changes in brain weights, enzyme activities and morphology. Thirty-five day old animals ex-

Table 3. Specific activity of choline acetyltransferase, glutamate decarboxylase and aromatic amino acid decarboxylase (means ± SD) in cerebellum of 35 days old piglets following prenatal and postnatal administration of trichlorfon.

		Cerebellum (nmol/h/g wet wt.)			
		Age (days)	Choline acetyl-transferase	Glutamate decarboxylase	Aromatic amino acid decarboxylase
Experiment 1 (sow A)	Postnatal medication	35	108 ± 10[a]	12868 ± 1534[c]	132 ± 28[e]
	Controls	35	110 ± 18[a]	12152 ± 2512[c]	116 ± 20[e]
Experiment 2 (sow B)	Prenatal medication	35	164 ± 12[b]	15070 ± 1930[d]	190 ± 8[f]

ab,ef: $p < 0.001$
cd: $p < 0.05$

posed prenatally, showed a significant increase in brain weights and enzyme activities as compared to piglets examined perinatally. The animals did not, however, reach control values in cerebral weight, cerebellar weight and total enzyme activity. Morphological changes included periodic loss of Purkinje cells in the cerebellum.

Several reports have demonstrated congenital ataxia and tremor in addition to a severe effect on cerebellar weight in piglets born to trichlorfon-treated sows (*Kronevi* 1977, *Kronevi & Bäckstrøm* 1977, *Bølske et al.* 1978, *Kronevi & Lindquist* 1978, *Knox et al.* 1978, *Fatzer et al.* 1981, *Gamlem et al.* 1983). *Berge et al.* (1987) observed not only a decrease in cerebellar weight, but also a severe reduction in the weight of the cerebrum of newborn piglets (67% of the controls). No ataxia and tremor occurred in the piglets even after repeated postnatal administrations. Furthermore, there were only small changes in the brain structures. This clearly indicates that the pig brain is less vulnerable to trichlorfon in the postnatal period of development. The cerebrum and cerebellum, which presumably were markedly reduced at birth (Exp. 2), increased in weight during the postnatal period. Although the increase in total weight (1.99 g) was less than in the control piglets (3.29 g), the percentage increase from term was similar in both groups.

Figure 1. Photomicrographs of sections from cerebellum of piglets 35 days old.

A - C: Normal control

D - F: Prenatal administration with trichlorfon. The only differences as compared with controls are a less well developed external granular layer and more frequently occurring areas with lack of Purkinje cells (E).

G - H: Postnatal administration with trichlorfon. No clearcut morphological differences as compared to controls.

Abbreviations: e.gr. - external granular layer
gr. - granular layer
mo. - molecular layer
p. - Purkinje cell layer

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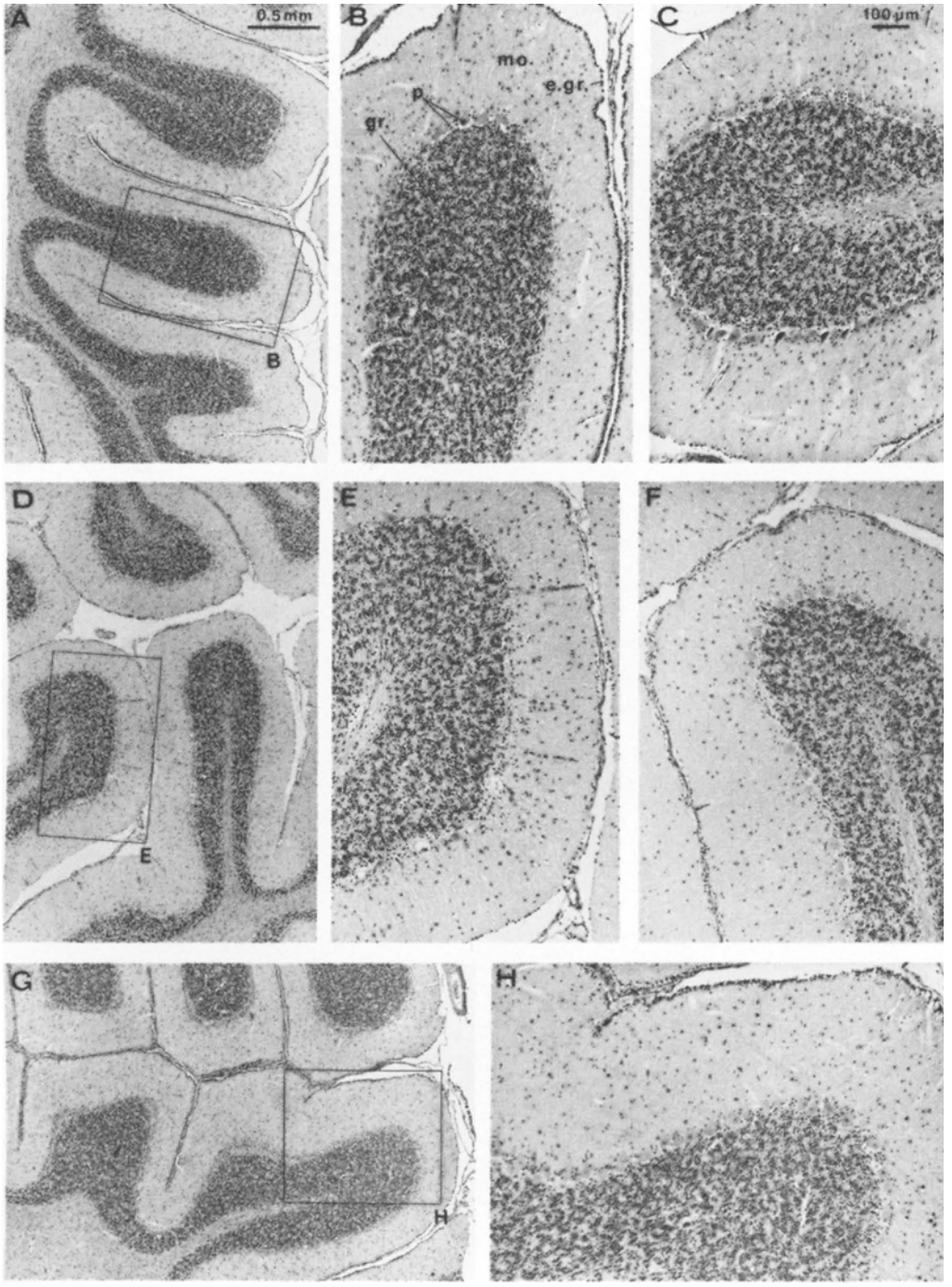

The transmitter enzymes ChAT, GAD and AAD in the cerebellum of 1-3 days old piglets have previously been found to be significantly reduced following prenatal administration of trichlorfon (*Berge et al.* 1987). The total activity of the same enzymes examined 35 days postnatally, was only slightly reduced (Exp. 1).

In Experiment 2, in which postnatal regeneration was examined, there was a decrease in total enzyme activities for all three enzymes compared to the control animals. Thus the structures producing these enzymes had only partly recovered. Specific enzyme activity was higher in the experimental group than in the control. It therefore seems that the structures producing the enzyme activity have increased faster than other structures in the cerebellum (i.e. granule cells, glial cells etc.). The most significant morphological finding in the cerebellum following prenatal medication is a regional loss of Purkinje cells (*Gamlem et al.* 1983, *Berge et al.* 1987). Trichlorfon, however, did not affect the distribution or number of Purkinje cells when administered in the postnatal period. The piglets in the regeneration experiment showed areas with lack of Purkinje cells in addition to a less developed external granular layer compared to normal piglets examined at birth.

The general sequential pattern of mammalian brain growth consists of two periods of rapid cell proliferation during development, the first being characterized by neuronal multiplication and the second by glia cell multiplication (*Dobbing 1972*). This sequence was also described in the pig by *Dickerson & Dobbing* (1967), who pointed out that the period of maximum growth rate could be divided into two consecutive parts, an early phase of rapidly increasing cellularity with its peak value occurring *before* birth and a later one of rapid deposition of lipid with the peak value occurring *after* birth. The same pattern was observed in both the brain as a whole and in the cerebellum. It seems likely that the defect caused by trichlorfon in the brain of the pig is associated with the earlier growth spurt of cell multiplication rather than to the later process of myelination.

We can, however, not exclude the possibility that prenatal as opposed to postnatal administration of trichlorfon may cause brain damage in the pig by preventing the transport of essential nutrients across the placenta at certain periods of gestation.

Acknowledgement

The authors wish to express their sincere thanks to professor Per Brodal for useful help with the histological examination and to professor Inger Nafstad for professional assistance, valuable advice and comments.

This work was supported by grants from the Norwegian Agricultural Research Council.

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Sammendrag

Nevrotoksikologisk undersøkelse av hjerne hos spedgris etter prenatal og postnatal administrasjon av triklorfon.

Spedgriser ble gitt 10 gjentatte doser med organofosfatet triklorfon postnatalt for å undersøke effekten på hjernens utvikling (Eksperiment 1). Etter prenatal administrasjon av triklorfon ble regenerasjonsevnen til en presumptiv hypoplastisk cerebellum og cerebrum undersøkt postnatalt (Eksperiment 2).

Administrasjon av triklorfon postnatalt resulterte utelukkende i små forandringer i hjernevekter, morfologi og transmitter enzymaktiviteter (kolin acetyltransferase, glutamat dekarboksylase, aromatisk aminosyre dekarboksylase) hos 35 dager gamle grisunger. Dyr som ble eksponert prenatalt, men ikke postnatalt, viste en signifikant økning i hjernevektene og enzymaktivitetene 35 dager post partum. Kontrollverdiene av cerebrumvekter, cerebellumvekter og totale enzymaktiviteter ble imidlertid ikke oppnådd hos disse dyrene. Stedvis tap av Purkinjeceller i cerebellum ble fremdeles observert.

Undersøkelsen viste at hjernen hos gris er lite sårbar overfor triklorfon i den postnatale periode av utviklingen sammenlignet med prenatalt.

(Received March 16, 1987).

Reprints may be requested from G.N. Berge, Department of Pharmacology and Toxicology, The Norwegian College of Veterinary Medicine, P.O. Box 8146 Dep, N-0033 Oslo 1, Norway.