Neuropathology of Experimental 3-nitro-4-hydroxyphenylarsonic Acid Toxicosis in Pigs

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Abstract. Twenty pigs were fed a diet containing 187.5 mg kg⁻¹ of 3-nitro-4-hydroxyphenylarsonic acid (3-nitro). Ten pigs were euthanized at intervals up to 29 days, 3-nitro was withdrawn from the diet of the remaining pigs on day 30, and these animals were subsequently euthanized at intervals up to 49 days after commencement of the experiment. A nervous syndrome characterized by clonic convulsive episodes inducible by exercise, developed at day 11. Paraparesis was apparent at day 22 progressing to paraplegia by day 33 (3 days after cessation of 3-nitro feeding). Histopathologic examination revealed myelin and axonal degeneration in the white matter of the spinal cord coincident with the onset of nervous signs. Marchi-positive degeneration was present in the dorsal funiculus at cervical level at day 22. Lesions intensified with increasing duration of toxicosis and while degenerate fibers were seen in all funiculi, there was preferential involvement of the fasciculi gracilis and cuneatus, the peripheral regions of the ventral and lateral funiculi, and a discrete area of the dorsal region of the lateral funiculus. Peripheral and optic neuropathies were evident from day 32 but were always mild and focal. The experiment establishes 3-nitro as a central-peripheral neurotoxicant of pigs.

Phenylarsonic acid derivatives have been used for many years for growth promotion and the control of enteric disease in pigs. The two most commonly used compounds are p-aminophenylarsonic acid (arsanilic acid) and 3-nitro-4-hydroxyphenylarsonic acid (3-nitro). Clinical and pathological aspects of both spontaneous and experimental arsanilic acid toxicoses have been previously described. However, it was not until recently that a previously unrecognized clinical syndrome of pigs characterized by convulsive seizures was described and demonstrated to have been caused by 3-nitro intoxication. This syndrome differed from arsanilic acid toxicity but resembled a previously described condition of unknown etiology and a disease attributed to others to a possible interaction between enterovirus infection and 3-nitro feeding. Subsequently, two further spontaneous outbreaks of 3-nitro intoxication in pigs have been recorded.

Earlier experimental toxicopathologic studies of the effects of 3-nitro administration to dogs, rats, mice, and chicks have concluded that the compound does not produce microscopic pathology even at subacutely lethal dose rates. Renal and hepatic changes were noted in a study of intoxication in lambs. However, these studies failed to include a histopathologic examination of the nervous system, and the only report of experimental neuropathology associated with the compound appears to be a description of peripheral neuropathy in turkeys.

Despite the continued and widespread use of 3-nitro as a feed additive, we could find no report in the literature of the clinical and neuropathologic aspects of experimental toxicity in any mammalian species. The experiment reported in this paper was designed to investigate and elucidate the sequential clinical events and spatio-temporal evolution of the neuropathologic changes occurring in 3-nitro toxicosis in pigs.

Materials and Methods

Twenty-four weaner pigs were randomly allocated to an experimental group of 20 pigs and a control group of four pigs. Both groups were fed a basal commercial pig grower ration and had access to water ad libitum. 3-nitro (Roxarsone, Salsbury Laboratories) was added at a rate of 187.5 mg kg⁻¹ to the diet of the experimental group from the commencement of the experiment (day 0) until day 30. Each pig was observed daily both at rest and after a few minutes forced exercise, and visual acuity was tested by estimating the ability to follow a moving object.

Two members of the experimental group were euthanized by intravenous injection of pentobarbitone sodium followed by exsanguination on each of days 5, 11, 15, 22, and 29. Following withdrawal of 3-nitro from the ration on day 30, the remaining pigs were euthanized on days 32 (2 pigs), 34 (1 pig), 35 (2 pigs), 39 (1 pig), 43 (2 pigs), and 49 (2 pigs). Control pigs were killed at the end of the experiment.

Following euthanasia, the entire spinal cord was exposed, and 10 cm lengths from each of the cervical, thoracic, and lumbar regions with corresponding dorsal root ganglia and 4 cm lengths of cranial and peripheral nerves (optic, left vagus at the level of the thoracic inlet, radial at its origin from the
brachial plexus, lateral branch of the radial at the level of the carpus, ischiatic at the point of emergence from the greater ischiatic foramen, and branches of this nerve at the level of the hock joint) were gently removed with minimal tension. The brain and eyes were also removed and all tissues were immediately placed in 10% neutral buffered formalin. Following fixation the tissues were trimmed (discarding previously cut ends of spinal cord and nerves), dehydrated, and embedded in paraffin. Transverse and longitudinal sections of spinal cord, longitudinal sections of peripheral and cranial nerves and sections of dorsal root ganglia (alternate ganglia from C2 to L6), brain and eye were stained with hematoxylin and eosin (HE) for histopathologic examination. Selected sections of spinal cord and dorsal root ganglia were also stained with luxol fast blue-cresyl violet, luxol fast blue-Holmes silver and Mallory's phosphotungstic acid-hematoxylin. Transverse 2 mm thick blocks of formalin-fixed spinal cord from cervical, thoracic, and lumbar levels of each pig were prepared for processing by Swank and Davenport's modification of the Marchi method for degenerate myelin. Interpretation of results obtained with this technique was based on criteria established by a previous investigator. In particular, a reaction was considered positive only when it could be traced across serial sections and was consistent when the method was repeated twice on adjacent blocks of spinal cord.

Results

Clinical findings were essentially similar to previous descriptions of 3-nitro toxicosis and have been described in more detail in a separate paper. The earliest definite indication of neurotoxicity in the experimental group was the development of typical clonic convulsive seizures following exercise. These episodes which were characterized by sudden onset of generalized, involuntary muscle tremors were initially mild but progressively increased in severity to produce violent shaking of the entire body. Affected animals stood still and frequently attempted to retain balance by placing the nose on the ground. They made repeated attempts to lie down but correct positioning of the limbs for this maneuver was frequently impossible. Loss of voluntary muscle control induced a state of fear as indicated by excessive squealing. Less severely affected pigs eventually achieved sternal recumbency with the assistance of nose support while more severely affected animals either collapsed or slipped on the ground. Tremors usually ceased immediately when they lay down but could be induced again by lifting the animal to the standing position. No animal died during these seizures although by day 30, each remaining pig had experienced and recovered from at least ten to 15 seizures. These episodes were inducible in each pig from day 11 until day 31 (1 day after removal of 3-nitro from the diet). Paraparesis was evident from day 22 and progressed to paraplegia by day 33. All remaining pigs developed paraplegia at this time but did not subsequently recover despite the cessation of 3-nitro feeding. There was no indication of blindness in any animal, and the control pigs remained clinically normal.

No significant macroscopic changes were seen in any animal at necropsy. No significant lesions were seen on histopathologic examination of the cranial or peripheral nerves of the control animals. Occasional myelin ovoids were however present in the white matter at all levels of the spinal cord in longitudinal hematoxylin and eosin (HE) sections. These figures were single and infrequent and were considered to represent either normal myelin turnover or processing artefact.

Distribution, time of onset, and relative severity of the neuropathologic lesions in the experimental group are indicated in Table 1. Isolated and single myelin ovoids were present in white matter at all levels of the spinal cord of the pig killed on day 5 and at thoracic and lumbar levels in the pig killed on day 11. These configurations occurred with a similar low frequency as in the control animals and were not considered sig-

Table 1. Spatio-temporal evolution of neuropathology in 3-nitro toxicosis.

<table>
<thead>
<tr>
<th>Region of Nervous System</th>
<th>Duration of Experiment (days)</th>
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<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Cervical spinal cord</td>
<td>Neg*</td>
</tr>
<tr>
<td>Thoracic spinal cord</td>
<td>Neg</td>
</tr>
<tr>
<td>Lumbar spinal cord</td>
<td>Neg</td>
</tr>
<tr>
<td>Radial nerve (proximal)</td>
<td>Neg</td>
</tr>
<tr>
<td>Radial nerve (distal)</td>
<td>Neg</td>
</tr>
<tr>
<td>Ischiatic nerve (proximal)</td>
<td>Neg</td>
</tr>
<tr>
<td>Ischiatic nerve (distal)</td>
<td>Neg</td>
</tr>
<tr>
<td>Optic nerve</td>
<td>Neg</td>
</tr>
<tr>
<td>Vagus nerve</td>
<td>Neg</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>Neg</td>
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* Neg = No lesions observed; 1+, 2+, 3+ = Lesions in ascending order of severity; ± = Equivocal lesions.
significant. Equivocal lesions were present at cervical level on day 11 but the first unequivocal lesions were not detected until day 15 (at cervical and thoracic levels). While initially myelin ovoids were single and occurred at a relatively low frequency, as the intoxication progressed they became more numerous and multiple with several ovoids often situated along the course of individual axons. Concurrent with the increase in myelin
ovoids, there was progressive contraction of myelin sheaths. Myelin shrinkage was associated with an increased affinity for eosin and resulted in the appearance of numerous unmyelinated segments of axons and progressive vacuolation of the white matter (Fig. 1). As the toxicosis progressed, lesions were also detected at lumbar level and generalized condensation and contraction of myelin resulted in all funiculi becoming increasingly eosinophilic and vacuolated. These changes were associated with little or no cellular reaction. More severe degeneration was characterized by fragmentation and loss of axons and complete destruction of myelin sheaths. These advanced changes were preferentially located in fasciculi gracilis and cuneatus and were associated with glial proliferation (Figs. 2, 3).

Low-level background staining was present in Marchi preparations of spinal cord from all control and experimental animals. This staining was apparently random, without localization, and was considered to be artefactual. Marchi-staining of the dorsal white funiculus was present in cervical cords of the experimental pigs killed at day 22 (Fig. 4). Degeneration was concentrated in the fasciculus gracilis and to a lesser extent in the fasciculus cuneatus. A similar but less intense, localized reaction was present at thoracic level, but the reaction of the lumbar cord, while considered to be positive in the dorsal funiculus, was weak. Only background staining was seen in ventral and lateral funiculi. By day 32, lesions in the cervical and thoracic cord had intensified and extended to include the entire fasciculus gracilis and a large proportion of the fasciculus cuneatus. Concurrently, a distinct localized reaction developed in the dorsal region of the lateral funiculus, lateral to the dorsal horn, at thoracic and lumbar levels (Figs. 5, 6). From day 43, a similar locus of degenerate fibers was identified in the cervical cord. A narrow band of Marchi-staining was seen in the peripheral region of the lateral and ventral funiculi at lumbar level on day 34 (Fig. 6). Degeneration of these tracts became more severe and was apparent at thoracic and cervical levels on days 35 and 43 respectively but was at all times much less intense than in the dorsal funiculus. As the duration of intoxication increased, the Marchi reaction intensified and degenerate fibers were apparent in all funiculi of the white matter at all levels of the cord. The progression of degeneration, as indicated by this technique, is represented schematically (Fig. 7).

Peripheral neuropathy, evidenced by condensation of myelin with formation of ovoids situated within the neurilemma, and axonal fragmentation (Fig. 8) was seen in the radial and ischiatic nerves of pigs killed on day 32. These focal lesions were infrequent and detected only after prolonged examination of longitudinal serial sections. The changes were indistinguishable from Wallerian degeneration and previously described arsanic acid neuropathy.6,9 While there was a temporal increase in lesion frequency, the incidence was
Fig. 5. Marchi preparation of thoracic cord (day 34). Intense staining of fasciculi gracilis and cuneatus. Discrete area of localized degeneration in dorsal part of lateral funiculus. (Background staining throughout all funiculi.) Bar = 1 mm.

Fig. 6. Marchi preparation of lumbar cord (day 49). Degeneration of a large part of the dorsal funiculus, in dorsal part of lateral funiculus and in periphery of lateral and ventral funiculi. Bar = 1 mm.
at all times low. No quantitative difference between proximal and distal regions of each peripheral nerve was appreciated.

Significant lesions were not detected in the optic nerve before day 32. From this time onwards, occasional myelin droplets were seen in vacuolar spaces. Phagocytic cells were often seen in close proximity to these droplets. There was no evidence of axonal degeneration, and lesions were at all times mild.

No lesions were present in the eye, brain including gracile and cuneate nuclei, vagus nerve, or dorsal root ganglia of any pig.

Discussion

Clinical signs and neural lesions of arsanilic acid and 3-nitro-4-hydroxyphenylarsonic acid (3-nitro) toxicoses in pigs have previously been considered as organoarsenical toxicity. The present experiment clearly demonstrates, however, that 3-nitro intoxication produces a clinicopathologic syndrome significantly different from reported descriptions of arsanilic acid intoxication. Paralysis and blindness associated with p-aminophenylarsonic acid intoxication may be explained on the basis of peripheral and optic neuropathies, but the convulsive episodes produced when the p-aminomoiety of this compound is replaced by the 3-nitro and 4-hydroxy groupings require a different explanation.

A good temporal correlation existed between the appearance of spinal cord lesions (11 to 15 days) and the onset of convulsive seizures in this experiment. Degeneration commenced in the dorsal white funiculus at cervical level extending caudad as the intoxication progressed. Little information exists regarding the arrangement of nerve fiber tracts within the spinal cord of the pig, but considering the results of one such study and the conventional understanding of the arrangement of the major nerve fiber tracts in the mammalian cord, it is apparent that the distal ends of the long ascending fibers of the fasciculi gracilis and cuneatus are preferentially damaged in 3-nitro toxicosis. It is also of interest that these fasciculi contain the fibers of

Fig. 7. Schematic representation of spatio-temporal evolution of spinal cord degeneration in 3-nitro toxicosis based on the Marchi technique. Degeneration proportional to degree of hatching.

Fig. 8. Ischiatic nerve (day 49). Destruction of a single nerve fiber with loss of axon and formation of myelin ovoids containing axonal fragments (arrow). HE. Bar = 100 μm.
mediation of proprioceptive impulses from muscle spindles, tendons, and joints to higher centers in the medulla oblongata. It is tempting to speculate that interference with propagation of these impulses may account for the convulsive seizures.

Several other clinical features in the present experiment and in a report of spontaneous toxicity support this hypothesis. During each convulsive episode, the pig remained conscious, was in an obvious state of fear, and could actively use the snout as a “fifth limb” while electroencephalographic patterns appeared normal. These observations coupled with failure to see brain lesions tend to support the hypothesis that major neuropsychological derangement occurs at a level below the brain. Of interest also is the fact that, except in more advanced stages of intoxication, convulsions ceased when the pig achieved sternal recumbency. This may be because proprioceptive information from the trunk and limbs is of less importance for awareness and maintenance of “position in space” in the recumbent animal. However, the present report cannot validate this hypothesis, and no explanation can be offered for the fact that while spinal cord degeneration is obviously continuous, the convulsions are intermittent, precipitated only by exogenous stimulation such as forced exercise. Definition of this physiological trigger mechanism will require further research. Furthermore, since convulsions could only be induced during and up to 24 hours after 3-nitro feeding, it appears that in addition to spinal neuropathy, relatively high tissue arsenic levels may be a prerequisite for the onset of these episodes.

In contrast to the progression of degeneration in the fasciculi gracilis and cuneatus which commenced at cervical level, degeneration of fibers in the peripheral regions of the ventral and lateral funiculi and in the dorsal part of the lateral funiculus appeared to commence at lumbar level extending to higher levels at a later stage. It would be of interest to know whether the component neurons of these tracts are ascending or descending and hence whether 3-nitro toxicosis produces a distal axonopathy in these tracts as in the fasciculi gracilis and cuneatus.

Selective involvement of the dorsal white funiculi has been reported in a description of spontaneous 3-nitro toxicosis. These authors suggested that some pigs may develop and recover from a convulsive episode without developing neurologic lesions. However, in the present experiment, spinal neuropathy was seen in all pigs which had undergone a convulsive episode with the exception of the pigs killed on day 11 in which cases unequivocal lesions were seen. It is possible that convulsions are always preceded by spinal neuropathy but that failure to observe lesions may be due to the difficulty in detecting early neuropathologic changes in the spinal cord.

Peripheral nerve lesions were at all times mild and focal in nature and even in paraplegic pigs did not approach the severity of the lesions described for 3-nitro toxicosis in turkeys fed 100 mg kg\(^{-1}\) of 3-nitro. These authors found evidence of a peripheral “dying back” neuropathy, but no such quantitative difference was appreciated between the proximal and distal regions of the peripheral nerves examined in the present experiment. Peripheral neuropathy resembled previous descriptions of arsanilic acid intoxication but was apparently of a much lesser severity. Mild optic neuropathy was also in marked contrast to arsanilic acid toxicosis.

The present report confirms that the clinical and pathologic features of 3-nitro and arsanilic acid toxicoses in pigs differ significantly. 3-nitro toxicosis produces a pathognomonic clinical syndrome and histopathologic confirmation of intoxication should be based on examination of the spinal cord and, in particular, the dorsal funiculus at cervical level.

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