Acta vet. scand. 1974, *15*, 533—543.

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THE TOXIC EFFECTS OF DIMETHYLNITROSAMINE IN SHEEP

By

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KOPPANG, NILS: *The toxic effects of dimethylnitrosamine in sheep.* Acta vet. scand. 1974, *15*, 533—543. — Experimental feeding, dealing with long term ingestion of small daily doses of DMNA has clearly demonstrated the cumulative toxic effect of this compound in sheep. A threshold level, somewhere between 0.10 mg and 0.15 mg DMNA/kg body weight per day, can be defined below which no toxicity even in quite long-lasting feeding can be demonstrated. Above this level toxicity changes will develop, clinically in most cases, anatomically in all, if the exposure time is long enough. If the daily intake of DMNA is over this threshold level, 21—40 mg DMNA/kg body weight, it usually causes liver disease and death in sheep. Neoplastic lesions were not produced, the experimental period, however, was below the latency period for carcinogenesis.

sheep; liver; dimethylnitrosamine; toxic threshold.

Dimethylnitrosamine (DMNA), a compound formed in certain animal feeds during processing, may lead to progressive liver damage in animals fed such feeds (herring meal) (*Koppang* 1962, 1964, 1966, 1967, 1970). Small amounts of DMNA, however, not only occur in fish and fish products (*Ender & Ceh* 1967, *Fazio et al.* 1971, *Fong & Walsh* 1971), but are also formed during the processing and storage of certain meat products, cheeses (*Möhler & Mayrhofer* 1968, *Fiddler et al.* 1971, *Sen* 1972, *Crosby et al.* 1972, *Wasserman et al.* 1972), beverages (*McGlashan et al.* 1968), and occur even in fruits (*DuPlessis et al.* 1969). The nitrosamines are formed by the interaction between nitrite, either naturally present or used as a preservative, and a wide variety of amines (*Wolff & Wasserman* 1972). The reaction even proceeds in the gastrointestinal tract (*Sander* 1967, 1971, *Sander et al.* 1968, *Sander & Bürkle* 1969, *Klubes et al.* 1972) and the vagina

(*Harrington et al.* 1973) mediated by bacteria. Although nitrosamines have not yet been found or actively sought in untreated sewage or soil, potential has been indicated for their formation in such environments (*Ayanaba et al.* 1973).

Attention to the toxic effect of DMNA was rapidly eclipsed by interest in the fascinating carcinogenic effects of these compounds, discovered by *Magee & Barnes* (1956).

In view of the small quantities in which this material is found, a study of the long range toxic effect of small daily doses seemed appropriate. The current experiments, dealing with long term ingestion of small daily doses of DMNA, not only explore the toxic effect but establish a toxic treshold in addition.

MATERIAL AND METHODS

The experiments were performed in the latter part of 1967 and the earlier part of 1968 at the Research Farm of the Veterinary College of Norway. Thirty-two female lambs of the Norwegian "Spelsau" breed, obtained from different sources, were used. They were kept at the Research Farm for two weeks for adjustment prior to the commencement of the experiment. The animals then received a dose of toxic herring meal added to their diet. The DMNA content in the herring meal was determined by the polarographic method (*Lydersen & Nagy* 1967). Four test groups of five animals each were formed, based on dosages of 0.50, 0.25, 0.15 and 0.10 mg of DMNA per kg body weight per group respectively. The actual intake was considerably higher, however, since the determination of DMNA levels in the stored herring meal bags six months after the initial assay showed an increase in level from an average of 70 parts/million (p.p.m.) to 99 p.p.m. with a variation range from 74 to 120 p.p.m. This increase in level during storage was unexpected, and the exact amount of DMNA ingested by the animals is therefore not known. The table computes intake on an assumed mean of 96.67 p.p.m. DMNA for groups 1, 2, 3 (all having received herring meal from the same bags). Group 4 was fed from a different source, a bag stored for more than six months, with a stable content of DMNA of 88 p.p.m. This fourth experimental group (0.10 mg/kg) was not instituted until the animals from the first groups started to die and it became obvious that a lower dosage range had to be investigated.

There were three control groups of four sheep each, who received 200 g, 100 g, and 50 g of non-toxic herring meal in their feed. Other food, housing etc. were identical. The toxic herring meal was mixed with barley, total mixture 300 g/day. In addition, each animal received 500 g of hay.

Laboratory tests. All animals were subjected to venipuncture every second week. The serum was examined for the activity of glutamic oxalacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), lactate dehydrogenase (SLDH) and α-hydroxybutyratic dehydrogenase (SHBD) (*Tollersrud* 1970).

Chemical examination for DMNA. Livers from two sheep from groups 1 and 2, were analyzed for DMNA after *Ender & Ceh*'s method (1971).

Histological examination was performed on all animals, the tissues being fixed in 10 % formalin, paraffin embedded, and stained with hematoxylin-eosin, a combination of Weigert's resorcin fuchsin and van Gieson, a trichrome procedure and Wilder's reticulum stain as reported before (*Koppang* 1964).

RESULTS

Clinical data

Group 1 (0.50 mg DMNA/kg). The sheep fed normally during the first three weeks of the experiment. In the fourth week two of the sheep began refusing some herring meal, their intake decreasing steadily until they died on days 47 and 53. The remaining two sheep proceeded to feed normally, refusing some of their diet only in the last week, dying on days 58 and 62. The last animal started to refuse some of the meal in the ninth week of the experiment and for the next two weeks only about half of the ration was consumed. After this, intake returned to normal and this sheep remained healthy until sacrifice, 180 days after the onset of DMNA feeding. The total consumption of the five animals ranged from 27 mg DMNA/kg body weight to 107 mg in the case of the only surviving animal (ref. Table 1).

The animals appeared dull when they started refusing feed but did not otherwise show clinical symptoms until the last two days of life when they rejected all feed. The animals kicked the trough, groaning in pain. There was atonia of the fore-stomach, respiration was accompanied by grunting and in the final stages ataxia.

Group 2 (0.25 mg DMNA/kg). The first animal to begin refusing some of the feed did so in the fourth week, the other animals continued feeding until fairly near death. Four of the five

Table 1. Feeding DMNA-containing herring meal to sheep.

	Sheep no.	Mean body weight kg	Herring meal intake kg	No. of exper. days	mg DMNA/kg body weight per day (mean)	mg DMNA/kg body weight total	Pathologic changes	Remarks
	526	24	6.8	47	0.57	27	+++	died
	128	21	8.2	53	0.70	37	+++	„
Gr. 1	113	25	8.5	58	0.56	33	+++	„
	393	19	8.3	62	0.65	40	+++	„
	577	23	26.2	180	0.59	107	++	sacrificed
	580	22	5.1	63	0.35	22	+++	died
	136	23	5.7	72	0.34	25	+++	„
Gr. 2	62	27	5.9	73	0.29	21	+++	„
	304	19	6.1	84	0.35	30	+++	„
	553	28	16.9	180	0.32	57	+(+)	sacrificed
	585	25	5.1	103	0.19	20	+++	died
	371	30	5.3	107	0.16	17	+++	„
Gr. 3	991	23	5.6	111	0.21	23	+++	„
	552	26	5.9	118	0.18	22	+++	„
	475	20	9.1	180	0.24	44	+	sacrificed
	127	26	6.5	203	0.11	22	—	sacrificed
	133	28	6.5	203	0.10	20	—	„
Gr. 4	61	30	6.5	203	0.09	19	—	„
	152	27	6.5	203	0.10	21	—	„

+++ Typical picture af toxic hepatosis.
++ Moderate liver changes, initial toxic hepatosis.
+ Modest liver changes, „ „ „
— Normal picture.

died between 63 and 84 days (ref. Table 1). The last animal remained healthy until sacrifice on the 180th day at the conclusion of the experiment.

Group 3 (0.15 mg DMNA/kg). Four sheep died between 103 and 118 days showing the same symptoms as the animals in groups 1 and 2. The last sheep rejected some of the meal in the 17th—19th test week, but otherwise appeared healthy and was sacrificed at the end of the experiment at 180 days (Table 1).

Group 4 (0.1 mg DMNA/kg). These animals ate the entire ration offered to them, remained healthy and were sacrificed 203 days after the beginning of the experiment.

Control groups. All 12 sheep were healthy and gained weight normally.

Blood chemistry. The SGOT levels of the animals in the first group increased to more than double the normal level, up to 335 units after 14 days, levels kept increasing until death. One animal showed a slight increase to 280 units, then started rejecting feed, the SGOT level decreased to 100 units. On resumption of normal feed intake, SGOT level rose again. In the second group SGOT levels started to increase after 45 days and in the third group after 90 days, whereas the fourth group showed no meaningful increases. Lesser increases were seen in SGPT, SLDH and SHBD.

Chemical examination for DMNA. DMNA was indetectable in the two livers examined.

Pathological findings

Gross changes. The four animals that died in each of groups 1, 2 and 3 showed a similar picture. Nutritional state was average, some animals from the second and third groups were well nourished. There was pallor of the mucous membranes and there was slight jaundice in two animals out of the first group. A generalized bleeding tendency with petechial hemorrhages and ecchymoses as well as bleeding into various portions of gut (duodenum, cecum, rectum) were seen. There was enlargement of the lymph nodes, particularly abdomen and thorax, again often with petechiae. There was hemorrhage into the thymus and pancreas. The spleen was markedly enlarged, about fourfold, and there was obvious portal venous congestion. Some animals showed slight anasarca with transudation of a clear yellow or slightly blood-tinged fluid into the serous cavities, in the case of the abdomen up to 5 l, in case of the pleural cavity up to 1 l. Edema was quite evident in the plicae spirales of the abomasum. The lungs were heavy and edematous; there were no infiltrations. The heart was flabby, filled with poorly coagulated blood. The livers in the animals in group 1 that died spontaneously were markedly enlarged with tense capsule and rounded edges. The color was red-brown with some small yellowish brown spots; there was a moderate nutmeg pattern on cut section as well.

In the animals that lived longer, the morphological picture was somewhat different. The liver tended to become smaller with a firmer general texture and with thickening and scar-like retractions of the capsule. Diffuse atrophy of the liver with harden-

ing was particularly obvious in the third group. The surviving sheep of group 1, sacrificed after 180 days, showed marked localized fibrous atrophy, limited to the left and the caudate lobe. Apart from slight swelling and congestion there were no major changes in the other organs, including the brain.

Histological changes

The liver. In acute cases the picture was dominated by hemorrhagic centrilobular necrosis affecting most of the lobules with confluence between neighbouring lobules, thus leaving intact liver cells around the triads only. Some lobules have replaced central necrotic cells by proliferation of connective tissue (Fig. 1). Normal lobules were found side by side with changed lobules. The central and sublobular veins showed various changes. In the acutely necrotic areas, acute vessel changes appear as necrosis of the intima, edema and hemorrhage into the vessel wall. In the less acute areas, more protracted changes of the hepatic vein system occurred, such as thickening of the vessel walls and partial or total obliteration of the central and sublobular veins (Fig. 2). With decreasing daily dosage and increasing days of experimentation, the acute liver changes diminished while the chronic changes were more pronounced with formation of connective tissue in the central areas of the lobules, simultaneously the obliterating changes of the central and sublobular vessels were enhanced. In the two sacrificed sheep of groups 1 and 2 the chronic obliterating changes of the left and caudate liver lobes were particularly clear.

Regeneration of the hepatic parenchyma is seen by the presence of voluminous hepatocytes with large nuclei and prominent nucleoli. There was partial new formation of connective tissue in the periportal areas and a moderate proliferation of

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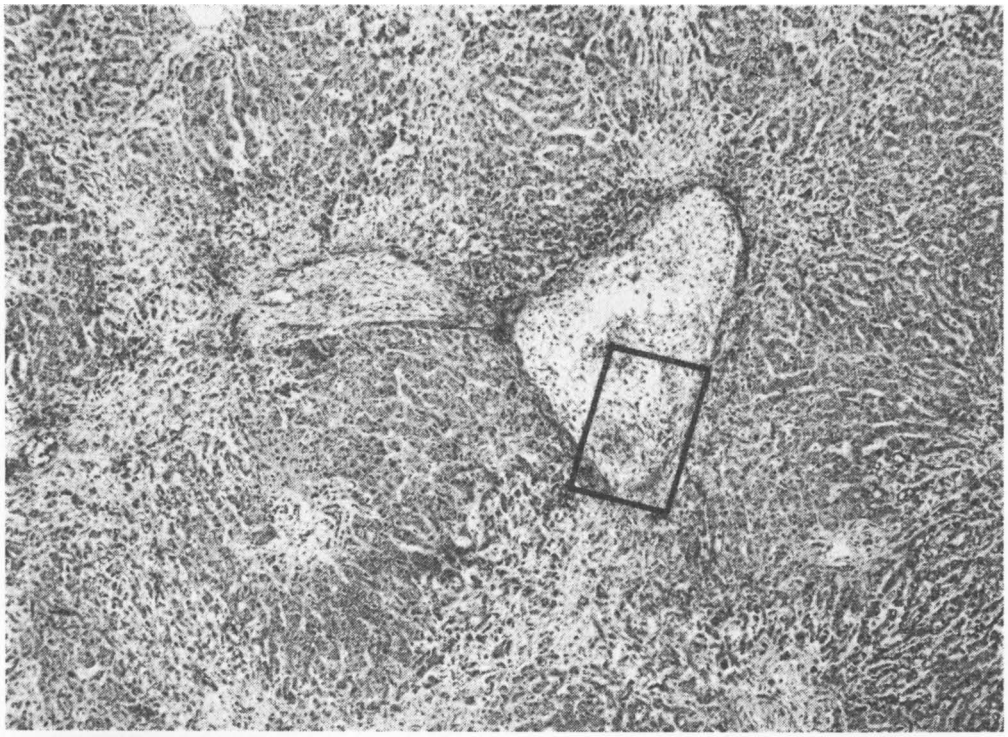
Figure 1. Liver from sheep group 1, which received 27 mg DMNA/kg body weight. Left: Confluent connective tissue proliferation centrilobular and across the interlobular division, leaving only islands of liver cells intact around the triads. In the center, sublobular veins with hemorrhage into the vessel walls and the surrounding liver tissue, proliferating fibroblasts are occluding the lumen of the two veins. Weigert — van Gieson, 68 ×.

Figure 2. Details of the square in Fig. 1. Proliferating fibroblast into the lumen of the hepatic vein. Weigert — van Gieson. 446 ×.



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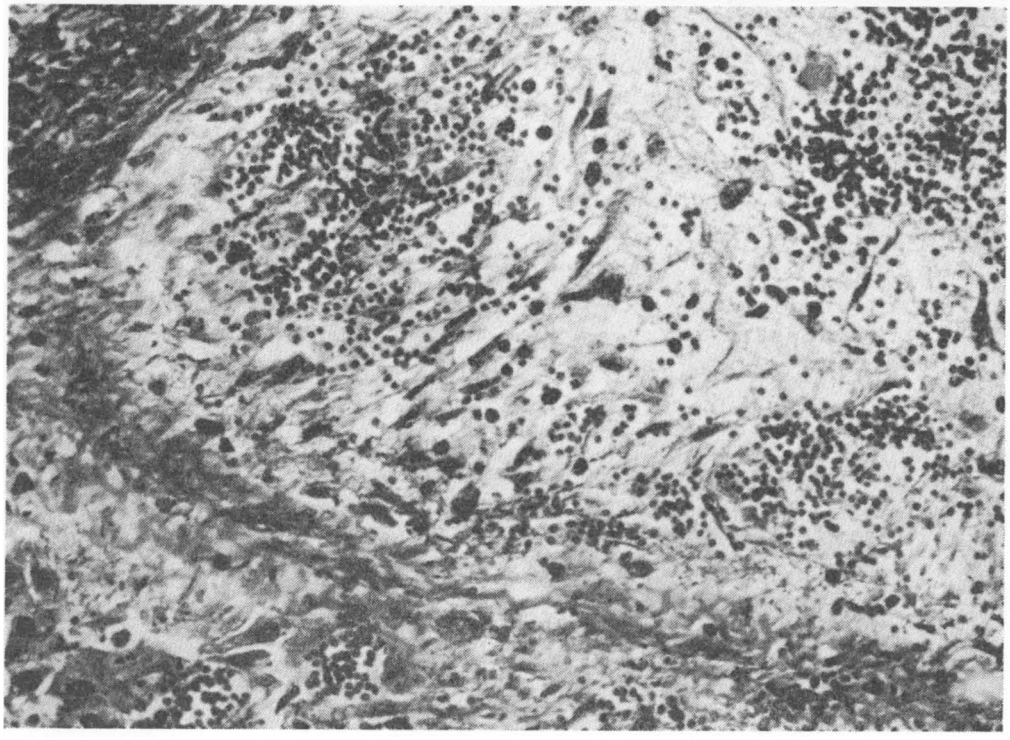

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some bile ducts. Pillow-like thickening of the wall of some major branches of the hepatic veins was seen.

The heart. Hemorrhage and edema of the muscle fibers frequently appeared together with a moderate infiltration of inflammatory cells, partly with degeneration of some fibers. Marked vascular lesions as seen in the liver could not be found, but in the more acute cases rather moderate damage to the vessels occurred.

The kidneys. Tubular epithelium in some nephron showed necrosis or degenerative changes in the distal convolute tubules and Henle's loop. Vascular changes were slight and of fibrinoid character. In the surviving animal out of the first high dosage group, some glomeruli displayed areas with increase in cellularity while others showed a slight fibrosis. Dilated tubules with a flattened and irregular epithelium could also be found.

The central nervous system. Minimal congestion with occasional minute hemorrhages was seen.

In the fourth group and in the control groups there were no histopathologic changes.

DISCUSSION

The total amount of DMNA ingested by the animals which died of DMNA intoxication was between 17 and 40 mg/kg body weight in all three groups. Surprisingly, in each group one animal survived, these animals were clinically normal as much as six months after exposure. The most extreme example is sheep 577 from the first group; this animal had received 107 mg DMNA/kg body weight or about five times the lethal dose for the other sheep and still showed no clinical signs when sacrificed although there were considerable hepatic changes at necropsy. When the SGOT level in this animal increased, the animal refused some of the diet returning to a normal intake after SGOT levels had dropped. Elevated SGOT, SLDH and SHBD levels have been found in sheep as well as cattle which were fed a non-toxic herring meal, that is to say free of DMNA (*Tollersrud* 1970), so that the specificity of these serum enzyme changes in terms of DMNA toxicity remains questionable. These latter animals had completely normal livers at necropsy.

Two other sheep had a greater than normal tolerance to the agent, 553 and 475 of the second and third groups which received 57 and 44 mg DMNA/kg body weight respectively. Most of the

liver tissue was normal although veno-occlusive changes were found mostly in the left and in the caudale lobes.

The animals in group 4 were normal both clinically and pathologically although the total amount of DMNA ingested was in the same range as that of animals in the other groups that died; a daily intake of 0.1 mg DMNA/kg body weight therefore must be considered below the threshold for a cumulative toxic effect of this agent.

It is clear that DMNA is a distinctly toxic substance and that the toxic effect is cumulative. Small repeated doses can result eventually in massive hepatic damage. It is interesting that, in sheep at least, a level can be defined (somewhere between 0.10 and 0.15 mg/kg) below which no toxicity even in quite chronic intake can be demonstrated and above which symptoms will develop, clinically in most, anatomically in all, if the exposure time is long enough.

In these experiments there was no evidence of development of any neoplasms, the experimental period, however, was below the latency period for carcinogenesis in sheep.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to G. Sand, Director of the Herring Oil and Herring Meal Industry's Research Institute, for processing the experimental meal and for part-taking in analytical work.

My thanks are also due to dr. S. Tollersrud for kind cooperation, and to Miss K. Harboe and Miss A. G. Grøthe for technical assistance.

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SAMMENDRAG

Toksisk virkning av dimethylnitrosamin på sau.

Foringsforsøk har vist at daglig opptak av dimethylnitrosamin (DMNA) har en kumulativ toksisk effekt på sau hvis dagsdosene er over en terskelverdi på ca. 0,1—0,15 mg DMNA/kg levende vekt, mens dagsdoser mindre enn denne terskelverdi ikke ga kliniske symptomer eller pato-anatomiske forandringer. Dagsdoser over terskelverdien forårsaket toksiske forandringer, spesielt i leveren, leverinsuffisiens og død når dyret har opptatt 21—40 mg DMNA/kg levende vekt. Svulster forekom ikke på forsøkssauene, men forsøkstiden var langt under den latensperiode som en må forvente for svulstdannelse.

(Received March 27, 1974).

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