Biochemical Investigations and Glyphosate Detection in Downer Cow Syndrome

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Abstract — A total of 258 dairy cows, 52 downer cows (fresh calving, resistant to therapies cows) and 206 apparently healthy cows at 14 dairy farms were investigated. Blood serum parameters as aspartate transaminase (AST), creatinine kinase (CK), urea, creatine, cholesterol and the trace elements as manganese (Mn), cobalt (Co), selenium (Se), copper (Cu) and zinc (Zn) were analyzed. Glyphosate was also tested in urine and feeds using ELISA. Downer cows had significantly higher CK ($p < 0.0001$), AST ($p < 0.0003$) and urea levels ($p < 0.05$) and significantly lower cholesterol ($p < 0.0001$) than healthy cows. The Co and Mn levels were generally too low in all animals based on reference standards. Downer cows excrete significantly less glyphosate in urine than their healthy comparisons which could be due to kidney damage. Significantly reduced cholesterol concentrations in the downer cows indicate that vitamin D3 is deficient and, with both Mn and Co deficiency, would negatively influence cellular immunity and increase the risk for rhabdomyolysis.

Index Terms: Trace elements, Downer cow syndrome, Biochemical parameters, glyphosate

1 Introduction

Downer cow syndrome (DCS) is an important condition of dairy cows which usually occurs following hypocalemic parturient paresis. Downer cows are recumbent cows that are not able to get up 24-48 h after calcium treatment [1,2]. DCS is a polymorphic disease characterized clinically by prolonged recumbency; however, the development of DCS varies. In 70% of DCS cases, splay leg and damage of muscles and nerves are the causes, 8-10% of cases are induced by myocarditis and hepatosis, 5 % suffer from persistent hypocalcemia, and 7% are of unknown etiology [3]. In Minnesota (USA) 2.1% of cows are reported to suffer from downer syndrome. Downer cows were high (48%) or average (46%) milk producers with only 6% to low milk producers. Approximately 58% with the disease manifest within 1 day of parturition with 37% occurring during the first 100 days of lactation. In New Zealand, the prevalence ranged from 3 to 5% of all dairy cows at calving time. In a clinical and laboratory survey of 433 periparturient recumbent cows in New Zealand, 39% recovered, 30% died and 32% were euthanized [4]. The case fatality rate was 11% higher in pre-calving recumbent cows than post-calving cows. The disease occurs most commonly in the first 2 or 3 days after calving in heavy milk producers and, in many cases, it occurs concurrently with parturient paresis.

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Prolonged recumbency (more than 4-6 hours) can result in ischemic necrosis due to obstruction of blood circulation, especially in a heavy cow if she lies on one leg for a long period. Experimentally, enforced recumbency of cattle for 6, 9 or 12 hours with one hind limb positioned under the body will result in downer syndrome. Affected cows are unable to stand, and the affected limb is swollen [5]. Persistent hypocalcemia and hypophosphademia may occur, but may not be the principal cause of DCS [6] although Barlet and Davicco [7] considered hypophosphademia one of the most important causes of DCS. One hypothesis is that the hypocalcemic state or ischemia due to prolonged recumbency may increase cell membrane permeability of muscle fiber and allow potassium to be lost from the cell to cause the myotonia which appears to be the basis of DCS [1,4].

Glyphosate (N-(phosphonomethyl) glycine) is a highly effective and important agronomic chemical that inhibits 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway available in plants, bacteria, fungi, algae and protozoa [8]. Inhibition of EPSPS prevents the conversion of phosphoenolpyruvic acid and 3-phosphoshikimic acid (S3P) to 5-enolpyruvyl-3-phosphoshikimic acid and shuts down the shikimate pathway, thereby inhibiting aromatic amino acid biosynthesis [9]. Glyphosate's herbicidal action is generated by chelating manganese required for the reduction of the flavin mononucleotide (FMN) co-factor of EPSPS [9]. Inhibition of the EPSPS is not the only activity of glyphosate. In warm blooded animals various other metabolic pathways are inhibited such as Cyp450 aromatase inhibition and genotoxic activity [10], teratogenic activity [11] and trace element chelation [12]. Some in vitro investigations with glyphosate have verified its cytotoxic effects on different cells at very low, sub-agricultural concentrations [13]. In a long-term investigation, Seralini and coworkers [14] reported significantly higher mammary tumor rates in female rats drinking glyphosate at 1 ppb, a very low concentration. Marked and severe kidney nephropathies and

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liver congestion were also reported. Glyphosate also can disturb the normal gut bacterial community in animals [15]. Originally glyphosate was patented as chelator to immobilize divalent cations. Later its herbicidal activity was detected and patented 1971. Genetic engineering technology inserted glyphosate resistance into genetically modified plants in 1996 that are grown extensively in North and South America [16]. The objective of the present study was to obtain information about the biochemical picture as well as the occurrence and possible health effects of glyphosate on downer cow syndrome at 14 dairy farms in central Germany.

2 Materials and Methods

2.1 Animals and sample collection

A total of 258 dairy cows, 52 downer cows (fresh calving, resistant to therapy cows) and 206 apparently healthy cows at 14 dairy farms were investigated. Downer cow samples were collected 6-48 h after detection of downer cow syndrome by the farmer. Downer cows did not respond to treatment with 500 ml Sorbivert® and C-B-Gluconat 24%®. Animals were injected again intravenously with 500 ml Sorbivert®, C-B-Gluconat 24%® and 20 ml Metaphosol® (Toldimfosnatrium-Trihydrat) without showing any progress. At the same time, 3% of apparently healthy cows of the same herd were sampled. The age of the cows was between 2 and 11 years. The average body weight of the cows was 550-600 kg. The number of collected samples from each farm and the farm history are shown in table 1. Blood specimens were taken from Vena coccygea mediana.

2.2 Investigation of blood serum for selected enzymes, electrolytes, vitamin B 12 and substrates

Blood serum parameters indicative of cytotoxicity (AST, CK), nephrotoxicity (urea, creatine), cholesterol and the trace elements (Mn, Co, Se, Cu and Zn) were analyzed by an accredited veterinary diagnostic laboratory (BioCheck GmbH, Holzhausen, Germany).

2.3 Glyphosate testing of urine

Also urine samples from all downer (N=52) and healthy (N=206) cows were collected for analysis of glyphosate excretion in urine using ELISA. Urine samples were diluted 1:20 with distilled water (aqua distillated, Braun, Germany) and tested for glyphosate by ELISA (Abraxis, USA) according to the manufacturer's instructions. Test validation was done with Gas Chromatography-Mass Spectroscopy (GC-MS) by Medizinsches Labor Bremen (Germany). The correlation coefficient between the two tests was 0.96 [17].

2.3 Glyphosate testing of feed

Two g of feed were transferred to 18 mL of distilled water, frozen over night at -20°C, thoroughly vortexed, and centrifuged at 3000 x g. The final dilution was 1:1000. Testing was with ELISA (Abraxis, USA) according to the manufacturer's instructions.

2.4 Investigation of the continuity of glyphosate excretion in urine per day

To investigate the continuity of glyphosate excretion in urine per day, fresh calving cows (2.5-6 years old at 550-600 kg) were investigated over a 24 h period at two farms. In each of the farms, two groups of three cows were involved. One group was fed ad libitum with the farm's normal mixed feed (NMF) while the other group was fed with the farm's own straw ad libitum. Urine samples were collected each 6 h for a total of 60 urine samples. The glyphosate concentration in straw and NMF was determined by ELISA.

2.5 Statistical analysis

The statistical program SPSS 15 (SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606) was used for data analysis. The recorded parameters were analyzed by the Shapiro-Wilkinson Test to confirm a normal distribution so that the arithmetic mean and standard deviation could be calculated. Median and 1st and 3rd quartiles were calculated for abnormally distributed values. Significance tests using Fisher's Exact Test and Mann and Whitney U Test were performed.

3. Results

3.3 Blood serum enzymes and electrolytes

The levels of CK, AST, and urea were significantly higher in downer cows compared with healthy cows, at $p < 0.0001$, $p < 0.0003$ and $p < 0.05$, respectively, (Fig. 1). Cholesterol was significantly lower at $p < 0.0001$ in the downer group (Fig. 1). Cu and Zn were significantly higher at $p < 0.01$ and $p < 0.04$, respectively, in downer cows compared with healthy cows (Fig. 2). The Co, Se and Mn levels were similar for the two groups. Co values in healthy and downer cows ranged from 0.30 – 0.56 µg/dL which were drastically lower than the reference values (102-203 µg/dL) (Fig. 2). Moreover, Mn values in healthy and downer cows ranged from 0.12 – 0.80 µmol/L which were drastically lower than the reference values (0.36-1.8 µmol/L) (Fig. 2). Se in healthy and downer cows was within the normal standards values (Fig. 2). There were no significant differences between vitamin B12 in both healthy and downer cows. The vitamin B12 values ranged from 126 - 410 (mean 226.56 ± 91.65) and 238 – 395 (mean 293.25 ± 73.15) ng/ml in healthy and downer cows, respectively.

Tabel 1

Characterization of investigated farms

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Farm No.	Milking cows	Total animals	Stillbirth frequency (heifers %)	Stillbirth frequency (cows %)	Culling rate %	Average downer cows/ month	Downer cows %	Main disease problem	Tested animals	
									Downer cows	Healthy cows
1	164	326	8	6	30	1	2	infertility	1	6
2	329	946	>5	>5	37	4	3	lameness	8	25
3	570	1254	5	6	35	5	5	lameness	6	9
4	164	394	8	10	25	5	10	infertility	9	15
5	64	211	16	5	25	3	10	infertility	1	4
6	662	1718	8	7	30	2	3	infertility	7	24
7	845	1932	9	4	31	4	2.5-4.5	no information	7	35
8	661	865	4	3	15	5	15	lamemess	2	43
9	94	219	>5	>5	20	1	2	mastitis	1	4
10	112	254	6	5	35	1	2	lameness	2	4
11	78	170	5	5	25	1	5	downer syndrome lactation4	1	4
12	166	313	>5	>5	30	1	>1	lameness	2	10
13	213	414	7	12	25	2	3	infertility	1	12
14	149	444	9	9	15	4	10	no information	4	11

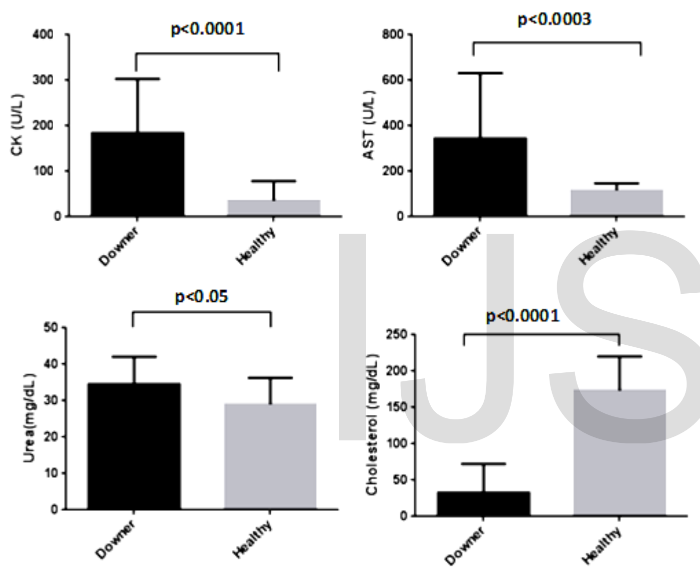

Fig 1. Enzyme levels in serum of downer and healthy cows. The reference values for CK, AST, urea and cholesterol are ≤ 100 U/L, ≤ 300U/L, 20-30 mg/dL, and >75mg/L, respectively.

3.1 Glyphosate levels in the urine of cows

Most of the cattle (either downer or healthy) excreted glyphosate in their urine (Fig. 3) with the range being from 1.3 to 164.1 ng/ml. Healthy cows excreted significantly ($p < 0.002$) more glyphosate in urine than downer cows. Glyphosate shedding in urine of downer cows (N=52) ranged from a mean of 0.0 to 164.1 ng/ml (mean 9.7) while it ranged from 1.08 to164.1 ng/ml (mean 23.3 ng/ml) in healthy cows (N= 206) (Fig. 3).

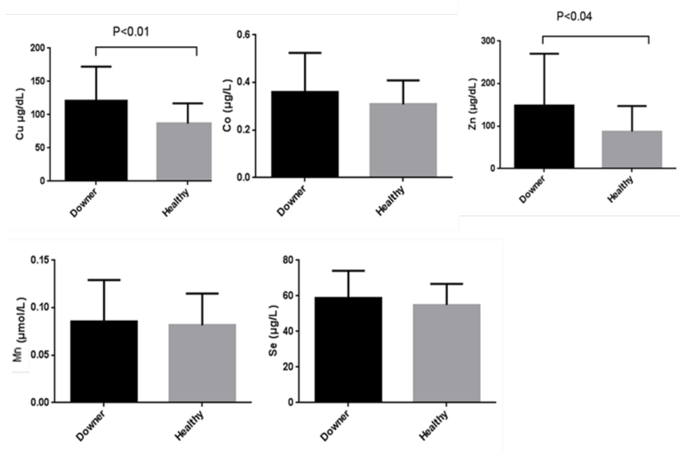

Fig 2. Trace elements in downer and healthy cows. The reference values for Cu, Co, Zn, Mn and Se are 102-203 µg/dL, 21.2-107.2 µg/dL, 70-130 µg/dL, 0.36-1.8 µmol/L and 70-100 µg/dL, respectively.

3.2 Continuity of daily glyphosate excretion in urine

Glyphosate excreted in urine of cows at two farms differing in glyphosate level in feed (farm 2 = low glyphosate level, farm 7 = high glyphosate level) in the course of a day is shown in figure 4. There were no significant differences in glyphosate excretion between the three cows of the straw group and the three cows of the NMF group at the two farms the whole day; however, the glyphosate level in urine between the two farms was significantly different ($p < 0.05$). The glyphosate concentration in farm 7's straw (114.7 ng/g) was 15.5 times higher than in farm 2 (7.4 ng/g). The glyphosate concentration in farm 7's NMF (1137.9 ng/g) was 5.9 times higher than in farm 2's (129.9 ng/g).

4 Discussion

DCS is an important disease for high yielding dairy farms. Our results showed significantly higher AST levels in downer cows which indicate muscle and liver damage (Fig. 1). These results are in accordance with Kheirabadi et al. [18] who found an increase in serum AST activity in downer cows. However, AST values are not liver-specific and is easily elevated in muscle damage [19]. To identify the origin of AST (muscle or liver), the CK was tested in downer cows and healthy cows. The increases of AST concurrent with high serum CK activity suggest muscle damage. The significantly higher CK levels of the downer cows could be a sign of this malady [20]. Cholesterol was significantly lower in the downer cow group in our study. Cholesterol is an important prestage of vitamin D3 that is generated in the skin with UV exposure. A reduced 7- dehydrocholesterol level in the skin reduces vitamin D3 synthesis [21]. Glueck and Conrad [21] believe that vitamin D deficiency leads to higher risk of rhabdomyolysis. Downer cows had significantly higher Cu and Zn levels; however Co, Mn and Se levels were similar in the two groups. Since these results were obtained from 14 different farms, it is implausible that feeding had influenced this result. Co and Mn levels, two critical minerals for liver function and detoxification of xenobiotics [23], were generally too low in all animals based on reference standards (Fig. 2).

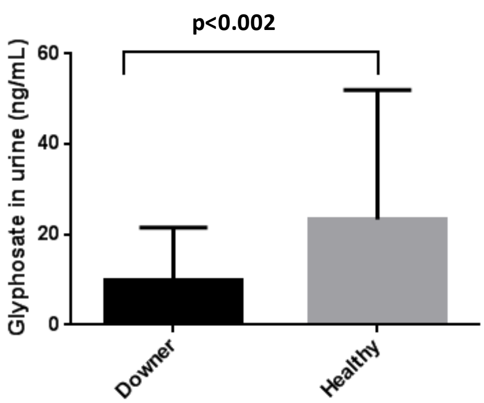

Fig 3. Glyphosate concentration in urine of downer (N=52) and healthy (N=206) cows.

Hence all tested animals either healthy or downer cows suffered from deficiencies of some trace elements as Mn and Co it was important to shed lights on the other causes of these deficiencies, like glyphosate. The extensive use of glyphosate containing herbicides world-wide has led to its wide-spread contamination of different ecosystems where it influences plants, microorganisms, animals and many components of the food chain. Moreover, glyphosate and its primary metabolite aminomethylphosphonate (AMPA) have been detected in immature seed [24] harvested seeds [25] and ground water [26]. World-wide, glyphosate is the most often used herbicide in intensive agricultural farming systems [27]. Treatment of numerous crops with glyphosate for pre-harvest desiccation, weed control in agriculture, home gardens, horticulture, and right-of-ways have led to increasing applications of glyphosate. Appendix II of the EU Commission of Health and Consumer Protection Directorate describes the metabolism of glyphosate after ingestion. About 30% of ingested glyphosate is absorbed, distributed in the body with low residues occurring in all tissues. Generally, glyphosate accumulation is below 1% after seven days [28]. Recently, Krüger et al. [29] documented the glyphosate shedding in urine and its impact on blood parameters in Danish cows. The authors found that glyphosate excretions in urine of cows were correlated with increased levels of GLDH, GOT and CK which demonstrate a possible effect of glyphosate on liver and muscle cells. Also high urea levels due to nephrotoxicity were correlated with glyphosate shedding. The very low levels of Mn and Co were observed in all animals which could be explained due to a strong mineral chelating effect of glyphosate.

In the present study, glyphosate excretion levels were tested in urine of healthy and downer cows. Our results show significant differences in glyphosate excreted in urine between downer and healthy cows (Fig. 3). Interestingly, the downer cows shed less glyphosate than the healthy cows. This indicates severe damage to kidneys as shown by Seralini et al. [28] in rats and by Gasnier et al. [30] for kidney cell lines at very low sub-agricultural dilutions of the herbicide Roundup 450 GT. The greater amount of glyphosate excreted by healthy cows of comparable performance and postural group indicate that healthy cows have healthier kidneys as also shown by significantly higher urea in the downer group (Fig. 1). Krüger et al. [29] reported there was a negative correlation between the amount of excreted glyphosate in urine and the creatinine level in blood serum. Although the glyphosate levels are not changed much during the course of a day at either of the two farms, based on 6 h urine samples taken from straw fed and NMF fed cows at each respective farm (Fig. 4), it was apparent that the amount of glyphosate in feed influences the amount excreted. Furthermore, it was noticeable that glyphosate excretion was relatively constant during the course of the day. Thus, it was not necessary to collect the samples at a specific time (Fig. 4).

The percentage of downer cows was between 2-15% in the farms investigated in this study (Table 1). Downer cows did not respond for treatment with 500 ml Sorbivert® and C-B-Gluconat 24%®. Animals were injected again intravenously with 500 ml Sorbivert®, C-B-Gluconat 24%® and 20 ml Metaphosol® (Toldimfosnatrium-Trihydrat) without showing any progress.

Barbosa et al. [31] proposed that glyphosate may contribute to neurological pathology by virtue of its chemical similarity with glycine, a co-factor required for activation of the N-methyl-d-aspartase (NMDA) receptor which controls excitatory actions of the central nervous system. NMDA receptors display sensitivity to an array of endogenous ligands and modulators present in the vicinity of the synapse that the co-agonist glycine must bind with before the receptors can be activated and physiological levels of protons suppress the activation of NMDA receptors. NMDA receptors have unusually slow 'activation/deactivation' kinetics [32]. Although glyphosate does not possess clinical NMDA receptor activity in acute poisoning [33], muscle fiber permeability could be influenced by glyphosate and its surfactant (adjuvants). Kim et al. [34] found cellular toxicity of the glyphosate-surfactant mixture impacted cell membrane integrity, metabolic activity, mitochondrial activity and the rate of total protein synthesis. Peixoto [35] reported that mitochondria treated with the herbicide Roundup increased membrane permeability and energy deficiency. In these studies, the observed damage was not from the active substance, glyphosate, in the formulated herbicide but was from the POEA surfactant. Mitochondria are provided with a variety of bioenergetic functions that are mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis that are impaired by the commercial glyphosate herbicide.

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4 Conclusion

Downer cows excrete significantly less glyphosate in urine than their healthy comparisons. The significantly higher blood serum urea levels are a prime indicator of kidney damage.

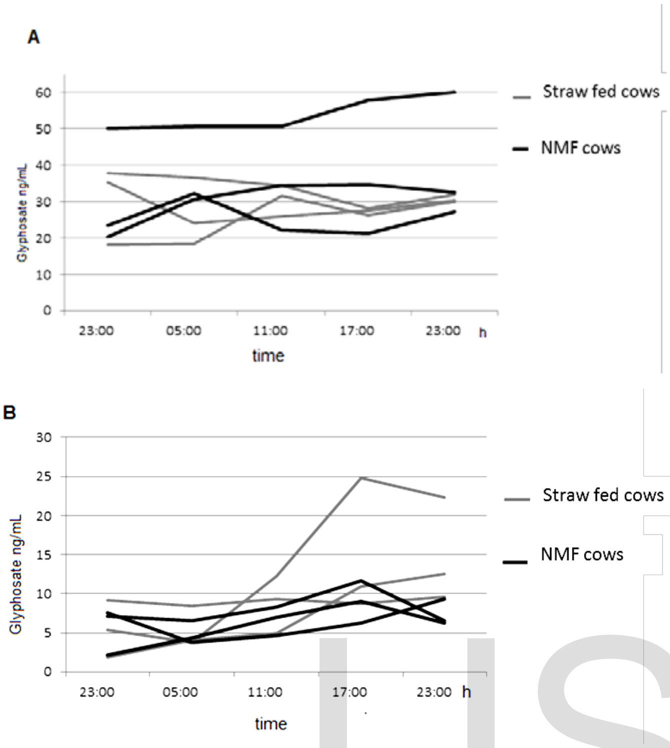

Fig 4. Glyphosate excreted in urine in the course of a day by straw fed (N=3) and NMF fed cows (N=3) at farms 7 (A) and 2 (B). Although glyphosate ingestion in A was much higher than in B, with one exception of a straw fed cow at farm 2 (B) and a NMF fed cow at farm 7 (A) there were no differences in glyphosate excretion during the course of the day.

High AST and CK blood serum levels also indicate impairment of liver and muscle cells common with DCS. Significantly reduced cholesterol concentrations in the DCS group indicate that vitamin D3 is deficient and, with both Mn and Co deficiency, would negatively influence cellular immunity and increase the risk for rhabdomyolysis. In conclusion, these results show that the generally ubiquitous glyphosate and its formulated Roundup herbicide might be considered in the pathogenesis of DCS.

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