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Brain in human nutrition and variant Creutzfeldt–Jakob disease risk (vCJD): detection of brain in retail liver sausages using cholesterol and neuron specific enolase (NSE) as markers

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No information is available about the consumption of brain via meat products. With respect to the new variant of Creutzfeldt–Jakob disease (vCJD) and the presumed food-borne transmission of bovine spongiform encephalopathy (BSE) to humans, a preliminary survey for brain and/or spinal cord (tissues of the central nervous system, CNS) was conducted. We applied a previously developed integrated procedure using cholesterol and neuron specific enolase (NSE) as markers. Quantification of cholesterol had to be backed up by NSE immunochemistry in order to account for low specificity and relatively high variances. Out of 126 high-quality finely graded liver sausages, five samples (4 %) showed positive NSE immunoresponses. In four of these samples a transgression of the normal maximum cholesterol content was obtained. The identification of such a considerable number of CNS-positive sausages indicates that brain consumption is not as rare as previously assumed. Overall, the present integrated method could be successfully applied for the detection of CNS in heat-treated meat products. Its routine application in official food control would deter illegal practice and thus help to control transmissible spongiform encephalopathies.

Brain in human nutrition: Meat products: Transmissible spongiform encephalopathies

Brain is assumed to be an extremely rare and exotic component in human nutrition. In 1957, the consumption of brain during ritualistic cannibalism was associated with the transmission of kuru, a human spongiform encephalopathy of some Fore tribes in Papua New Guinea (Gajdusek, 1977). The appearance after 1986 of new, presumably feed-borne, forms of transmissible spongiform encephalopathy (TSE) in bovines (BSE), in exotic ungulates, and in felines led to the assumption that humans might be infected via bovine brain entering the human food chain. This was substantiated when in 1996 the first 10 cases of a new variant of Creutzfeldt–Jakob disease (vCJD) were described (Will *et al.* 1996). It must be assumed that the greatest risk, if any, was posed by meat products produced with substantial amounts of bovine brain and/or spinal cord (tissues of the central nervous system, CNS): 1, Infectivity titres of bovine CNS exceed that of any other tissues up to several orders of magnitude (SSC, 1998); 2, weight of CNS (adult bovines: 630–740 g; Seiferle, 1992) exceeds that of the few other tissues showing high infectivity titres by at least 1 order of magnitude; 3, the use of CNS in the meat processing industry is of some interest considering its high content of substances with emulgating properties, high nutritional value, low price, and ready availability (mechanical deboning of skulls and vertebral column); 4, it is estimated that 840 000–1 250 000 infected animals were allowed to enter the human food chain, as BSE infectivity is present for a considerable time before the onset of clinical symptoms and the measures taken to enforce the ban were to some extent ineffective (Anderson *et al.* 1996). However, industry disclaims and, in many countries, law or food codes prohibit the addition of CNS to meat products. Whether brain and spinal cord of slaughtered animals were actually used in the manufacture of meat products during the critical period prior to the ban of specified bovine offal (UK Government, 1989) is a matter of speculation as methods for their detection were missing at that time. Recently, it was demonstrated that cholesterol and neuron specific enolase (NSE, Table 1) could be used as markers for CNS in heat-treated meat products (Lücker *et al.* 1999). These studies used mainly in-house reference materials with known addition of CNS and only a few field samples from local retail outlets. Thus, the present study was designed as a survey for CNS addition in retail meat products currently on the market in Germany. We chose high quality, finely ground cooked liver sausages as the use

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Table 1. Use, content and frequency of selected ingredients in meat products and contents of cholesterol (mg/100 g fresh product) and of neuron specific enolase (NSE, ng/mg soluble protein) as reported in literature

Ingredient	Use (content, frequency)	Cholesterol	NSE
Brain	Not allowed in most products[a] (1–20 %, ?)	2223±271[d]	11200±3200[c]
Spinal cord	Not allowed[a] (?)	2420±310[e]	13000±1000[c]
Dorsal root ganglia	Via mechanically deboned meat (?)	–	831–1145[b]
Optic nerve	Via mechanically deboned meat (?)	–	4200–7800[b]
Peripheral nerves	Technologically unavoidable (?)	–	59–765[b]
Muscle	Main component (20–100 %, obligatory)	69±7[d]	29±5[c]
Adipose tissue	Usual component (2–50 %, obligatory)	72±4[d]	47±25[c]
Heart	Some products (1–5 %, rare)	146±11[d]	40±21[c]
Liver	Liver sausages (10–30 %, frequent)	365±82[d]	8±5[c]
Blood	Blood sausages (10–20 %, frequent)	106±40[d]	189±14[f]
Kidney	Not allowed in most products[a] (1–5 %, rare)	376±12[d]	15±9[c]
Lung	Not allowed in most products[a] (1–5 %, rare)	239±72[d]	28±17[c]
Yolk	Not allowed in most products[a] (1–5 %, rare)	1260[d]	–

[a] German Food Code (1994), [b]Marangos *et al.* (1979), [c]Kato *et al.* (1982), [d]Souci *et al.* (1994), [e]Runquist *et al.* (1995), [f]serum, ?: unknown, –: not given; where possible mean and standard deviation are given as reported (including interspecies variations in[d]).

of CNS in these products would be of some technological advantage with respect to its emulgating properties. However, according to the German Food Code (2000), CNS is not allowed in the production of these sausages.

Materials and methods

Field samples

A total of 126 cooked liver sausages were obtained on the occasion of the 1998 quality testing of cooked products and lard by the German Agricultural Society (Hildebrandt & Kleer, 1998). All of the samples had a thoroughly homogeneous appearance (finely ground cooked liver sausages) and had been produced using moderate heating (storage stability: several days at ≤10°C). The sausages were of high quality which excluded the use of other offals besides liver according to the German Food Code (2000). The mode of sampling was totally randomised with manufacturer and provenance unknown to the sampling personnel. In addition, we used a further 76 samples as a negative control group. These included various types of cooked liver sausages: finely and coarsely ground; low to high quality; with and without addition of further ingredients such as onions. They were obtained from locally known and reliable butchers where the possibility of CNS addition could be excluded in all probability.

Reference materials

Reference material as used in this study was manufactured with defined addition of bovine brain following the usual recipe for cooked liver sausages. A basic filling was produced using a high-performance table-top laboratory cutting mixer (Diana-63004, Dianawerk, Kassel, Germany). The basic filling was composed of 1·0 kg pork trimmings from belly, shoulder and cheek (mean fat content: 40 %), 0·2 kg drinking water as substitution for the preboiling loss, and 0·02 kg curing salt (4–5 g/kg $NaNO_2$ in NaCl, Enders, Reiskirchen, Germany). The meat was preboiled at 90°C over 60 min, shortly cooled and processed at approx. 2900 rpm in the cutting mixer until a totally homogenous material was obtained. Hot water was added during the final part of homogenisation. Then the basic filling was homogenised with 16 % (or 15 %) of prehomogenised porcine liver and varying amounts (0·0, 0·25, 1·0, 2·0, 4·0) of prehomogenised bovine brain using a kitchen mixer (Moulinette, Kalensee, Gieβen, Germany). The material was filled into tins (150 ml) which were closed and heated for 60 min at 80°C in the steam heater. Reference material was analysed as control along with the field samples in both quantification of cholesterol and NSE immunochemistry.

Quantification of cholesterol

Quantification of cholesterol was achieved by use of the enzymatic testkit from Roche/Boehringer (Mannheim, Germany) for spectrophotometry (Anonymous, 1995); sample preparation and extraction in methanolic KOH were performed according to the guidelines as given for liver sausage. The test principle is based on the 3-β-oxidation of cholesterol to Δ^4-cholestenone. The resulting H_2O_2 oxidises methanol to formaldehyde in the presence of katalase. Subsequently a yellow lutidine dye is formed which is spectrophotometrically (Hitachi U2000) quantified at 405 nm.

NSE immunochemistry

Proteins were extracted from the sample matrix using tris-urea buffer at 4°C and a Potter–Elvehjem homogeniser (Novodirect, Kehl/Rhein, Germany), centrifuged at 14 000 rpm for 10 min, filtered, and diluted 15:1 (v/v) in sample buffer (tris–SDS–mercaptoethanol–urea). The proteins were separated by means of SDS gel electrophoresis using 10 % acrylamide gel (PAGE) according to Laemmli (1970) and then transferred on PVDF membranes (Millipore, Bedford, MA) using an electroblot apparatus (Biotec-Fischer, Reiskirchen, Germany). Following incubation in blocking solution (BS: phosphate buffered saline with 1 % (w/v) bovine serum albumin and 0·2 % (v/v) Tween 20, pH 7·4; Merck, Darmstadt, Germany) for 1 h, the membranes were incubated overnight at room temperature with monoclonal anti-NSE antibodies (Dako,

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Hamburg, Germany; clone BBS/NC/VI-H14). Then, the membranes were incubated with biotinylated goat anti-mouse antibodies (Zymed, San Francisco) diluted 1:2000 in BS over 1 h and then incubated with peroxidase-conjugated streptavidin (Zymed, San Francisco) over 60 min. Staining was achieved by adding H_2O_2 (Merck, Darmstadt, Germany) and DAB (3,3′-diaminobenzidine-tetrathydro-chloridhydrate, Aldrich, Milwaukee, USA).

NSE immunoresponse

Quantification of NSE immunoresponse was achieved by densitometric analysis (Scion Image, Release Beta 3b, Scion Corporation, Frederick, Maryland) of digitalised immunoblots. Peak area integrations were expressed in relation to the respective standard (non-heated liver sausage containing 1 % bovine brain) as analysed within the same immunoblot.

Computation

Statistical analysis of cholesterol data included the W statistic (Shapiro and Wilks' statistic), the two-group t-test and the non-parametric Mann–Whitney (Wilcoxon) rank-sum test; computation was performed using the programs BMDP 2D and 3D (Dixon, 1993). MS-Excel 97 was used for any further calculations. Cutoff points (x_{CP}) for the upper limits of normal maximum cholesterol content were calculated according to the following equation: $x_{CP} = \bar{x} + z \times s$, where $\bar{x}$ is the arithmetic mean cholesterol content of the samples and s the respective standard deviation in milligrams per 100 grams; z is the bounds of the standard normal distribution with $z = 1{\cdot}645$ ($P = 0{\cdot}05$, one-sided).

Results

Arithmetic mean ($\bar{x}$) and standard deviation (s) of the cholesterol contents of the 126 liver sausages were 123 and 31 mg/100 g fresh product, respectively. The results ranged from $x_{Min} = 59$ to $x_{Max} = 297$ mg/100 g with a median ($\tilde{x}$) of 120 mg/100 g fresh product. Two-thirds of the samples had cholesterol contents below 130 and 90 % below 156 mg/100 g fresh product. Thus, the distribution was found to be skewed to the left as shown in Fig. 1. The Shapiro and Wilkes' test gave a W statistic of 0·91 and showed a highly significant deviation from the normal distribution ($P < 0{\cdot}001$). Both approaches – iterative reduction of maxima until normality of distribution was reached and retrospective reduction of CNS-positive samples – yielded cutoff points for normal maximum cholesterol content ($P = 0{\cdot}05$) of 160 mg/100 g fresh product. Following elimination of samples presumably adulterated with CNS, the distribution of the cholesterol contents in liver sausages was characterised as follows: $\bar{x} = 119$, $s = 24$, $\tilde{x} = 120$, $x_{Min} = 59$, $x_{Max} = 181$ mg/100 g fresh product. Results were significantly lower ($P < 0{\cdot}001$, t test, Mann-Whitney test) than those obtained in the control group, where $\bar{x} = 150$, $s = 21$, $\tilde{x} = 151$, $x_{Min} = 92$, $x_{Max} = 186$ mg/100 g fresh product. The corresponding

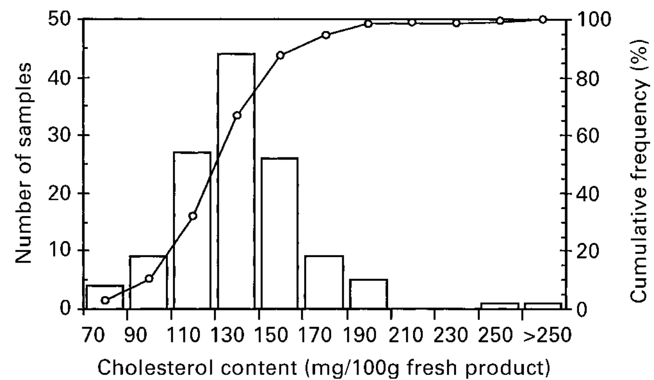

Fig. 1. Absolute and cumulative frequency of cholesterol in 126 cooked liver sausages as analysed by a colorimetric assay.

cutoff point for normal maximum cholesterol content in the control group was 187 mg/100 g ($P = 0{\cdot}05$). Results of the reference material (15 % liver, no brain addition) as analysed along with the field samples were $\bar{x} = 102$, $s = 5{\cdot}9$, and ranged from 92 to105 mg/100 g fresh product. The expected cholesterol content of the endproduct as calculated from the its components, the filling (90 mg/100 g) and the liver (274 mg/100 g), was 118 mg/100 g fresh product.

All results as obtained for the cooked liver sausage reference material (0, 0·25, 1, 2, 4 % brain) with SDS-PAGE and Western blotting, using monoclonal anti-NSE antibodies, were in accordance with previous findings, i.e. no false positive or false negative results were obtained and immunoreactivity was closely correlated to the amount of brain present in the sample. It was noted, however, that the intensity of the immunoresponses varied in some cases considerably between immunoblots. This is demonstrated in Fig. 2 which shows the 50 kDa NSE band for ten samples and their respective (non-heated) reference material with a brain content of 1 %. We obtained positive immunoresponses in five out of the 126 field samples (4·0 %). This was correlated in four cases with a transgression of the cutoff point for normal cholesterol content, whereas the cholesterol content of sample No. 45 was only 90·6 % of the cutoff point. The cholesterol content of 7 (5·6 %) samples transgressed the cutoff point of 160 mg/100 g with $\bar{x} = 172$, $s = 6{\cdot}4$, $x_{Min} = 164$, x_{Max}=181 mg/100 g fresh product. With respect to varying immunoresponses, the intensity of each sample band was calculated in relation to the band of the respective reference material (1 % CNS addition, non-heated) in the same immunoblot. The relative intensity of the immunoresponses (Fig. 2) proved to correlate moderately with the respective cholesterol contents ($r^2 = 0{\cdot}70$, with $y = 0{\cdot}0036\, x - 0{\cdot}46$).

Discussion

In contrast to the previous results obtained for emulsion type sausages (Lücker *et al.* 1999), the present study indicated that a cutoff point for the maximum normal

Sample no.	Cholesterol content (mg/100g)	NSE-Immunoblot Sample	NSE-Immunoblot Reference material	Relative intensity
95	97			0·000
5	104			0·000
46	145			0·153
96	146			0·000
60	150			0·000
64	159			0·000
92	177			0·328
36	182			0·037
21	245			0·239
51	297			0·819

Fig. 2. Cholesterol content in cooked liver sausages and detection of neuron specific enolase (NSE) in the western blot using monoclonal anti-NSE antibodies. The relative intensity is the densitometric intensity of the immunoresponses of the sample in relation to that of the respective reference material (non-heated sausage filling with 1 % addition of bovine brain) in the same immunoblot.

cholesterol content in cooked liver sausages cannot be safely established when combining the detection of low brain contents (<1·0 %) with high statistical security. In order to detect 1·0 % brain in cooked liver sausages, the cutoff point has to be drastically reduced, i.e. from 160 mg/100 g ($P = 0{\cdot}05$) to 140 mg/100 g ($P = 0{\cdot}20$). The low sensitivity might be a result of the additional variance due to varying amounts of liver and their varying cholesterol contents (Table 1). However, the results of NSE immunochemistry demonstrated cholesterol to be a suitable marker for CNS, albeit for screening purposes, especially if more sophisticated methods are not available. In Germany, this method has already been established in official food control for the quantification of cholesterol in egg and egg products (BgVV, 1992). The method itself had been described by Röschlau *et al.* (1974) long before the BSE crisis and thus could have been applied to support the ban of specified bovine offal. Pertaining to the relatively low specificity of the cholesterol quantification (Table 1), NSE immunochemistry is needed to confirm CNS adulteration of suspect samples. Reference material was correctly classified when NSE was used as a marker without exception, as was the case in previous studies (Lücker *et al.* 1999). This is of particular interest with respect to the presence of NSE in peripheral nerves found within muscle tissue (Table 1). In the present and previous studies, the reference material produced without addition of CNS never showed NSE immunoresponses, irrespective of composition (pure muscle or mixture of muscle and adipose tissue) and technology (heated or non-heated). Furthermore, with respect to preventive consumer protection, substantial amounts of peripheral nerve tissue are unwanted in meat products – even though BSE infectivity could not be demonstrated so far in this tissue. Thus a positive NSE immunoresponse due to peripheral nerves should not *a priori* be classified as a false negative result. Although there remains no doubt regarding the high specificity of NSE immunochemical detection of CNS in meat products, it is not clear whether sensitivity is adequate to account for possible reduction of NSE immunoreactivity due to technological effects, especially heat treatment. First results showing this to be the case (Lücker *et al.* 2000) emphasise the need for further studies specifically addressing the need for further studies regarding methodological optimisation.

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Conclusion

The detection of a considerable number of CNS-positive field samples indicate that the use of brain in meat products is neither rare nor exotic in Germany. The use of brain and/ or spinal cord in the production of sausages is in distinct contrast to the general expectation as founded on the lessons learned during the BSE crisis. It stands in further contrast to the disclaimer of the meat industry and to the prohibition by law. The general application of the presented methods in official food control might deter illegal practice and thus help to control transmissible spongiform encephalopathies.

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