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Analysis of the risk of transmitting bovine spongiform encephalopathy through bone grafts derived from bovine bone

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Abstract

Bone substitutes of bovine origin are widely used for treatment of bone defects in dental and orthopedic surgery. Due to the occurrence of BSE and the new variant of Creutzfeldt Jakob Disease risks of transmitting diseases through the use of such materials need to be carefully evaluated. Risk analysis can either be based on theoretical assessments or experimental evidence. Here we present a comparative study on two bovine bone substitutes (Bio-Oss® and Osteograf/N) which is based on theoretical values. Furthermore, for one of these materials, i.e. Bio-Oss®, the prion inactivation capacity of one of the production steps was experimentally evaluated. Theoretical and experimental data indicate that the use of these materials does not carry a risk of transmitting BSE to patients. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Bone substitute; Risk analysis; Bovine spongiform encephalopathy; Western blot

1. Introduction

Bone defects or inadequate bone volume represent a common problem in dental and orthopedic surgery. Bone substitutes have widely been used to restore and regenerate deficient bone. Bovine bone is a common source for preparation of bone substitutes. The manufacturing process of most of these bone substitutes include heating for several hours at temperatures above 1000°C transforming the material into a ceramics. However, such products may lose the unique microporosity of native bone and therefore their osteoconductive capacity may be reduced. Furthermore, the ceramics cannot be as well degraded as the non-sintered material and is therefore not resorbed properly [1]. These disadvantages can be overcome by heat treating the bone at maximum temperatures around 300°C allowing preservation of the bone structure [2].

Infectious particles (prions) cause Creutzfeldt–Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. Therefore, the use of bovine material for medical products and devices poses the question to what degree such material can be considered free of prions and what are risks of transmission of the disease to humans. There have been cases reported of iatrogenic transmission of CJD from humans to humans through the use of human-derived medicinal products [3]. While the appearance of the new variant CJD (vCJD) appears to be caused through consumption of infectious bovine food stuff, none of the vCJD patients had a history of surgery [4]. However, health authorities have set up regulations and requirements for minimizing risks associated with the use of bovine-derived pharmaceuticals and medical devices. The German health authority developed an internationally well-recognized risk assessment that is based on the statistical evaluation of relevant parameters associated with the production and application of bovine-derived products [5,6]. This assessment allows to evaluate the probability of transmission of BSE to humans through the use of a certain product.

Prions are considered to be composed mainly of an altered normal protein (prion protein). Denaturation or modification of proteins will lead to loss of infectivity. Therefore, experimental proof for prion inactivation through processing has become an important aspect for the safety. Recently, a Western blot technique has been

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described which allows the measurement of the inactivation capacity of certain production steps [7].

In this paper the safety of the bone substitute Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland; Osteohealth, Shirley, New York, USA) is evaluated experimentally as well as statistically. Bio-Oss® is prepared from US-derived bovine bone through a combination of chemical processing and heat that result in denaturation and removal of proteins and other organic substances. The mineral structure of Bio-Oss® remains mainly unchanged. The osteoconductivity of Bio-Oss® has been described in the scientific literature [8-10]. Recently, the results of a risk assessment of another bone substitute (Osteograf/N, CeraMed Dental, LLC, Lakewood, USA) was published by Sogal and Tofe [11]. As this publication was based on an older German Model from 1994 we repeated the calculation for this product according to the updated version from 1996 and compared the results to the ones achieved for Bio-Oss®.

2. Material and methods

2.1. *Statistical risk evaluation according to model of the German Federal Health Authority*

In order for minimizing risk of transmission of BSE or scrapie to humans through the use of medicinal products the German Federal Health Authority has published a system of safety requirements for products made from cattle, goat or sheep [5,6]. Risk evaluation includes all relevant aspects of production and application. As the probability of humans to get CJD spontaneously is less than one in a million, sufficient safety of a product can only be assumed if the probability of transmission is below 10^{-6}. The German risk assessment also induces the worst-case assumption that there are no species barriers between humans and animals.

Parameters for the risk evaluation of the German Federal Health Authority are: (1) origin and feeding of the animals; (2) type of tissue used for production; (3) processing steps for inactivation of prions; (4) amount of raw material needed for the production of one daily dose; (5) number of daily doses; and (6) method of application. Each of these parameters is classified according to a logarithmic scale which describes the statistical risk associated with each parameter. Higher numbers indicate lower risk of infection, i.e. increased safety. Factors of each parameter are added. The sum shows the increase in safety in orders of magnitude of the end product compared to the worst case. The sum has to be at least 20, since this corresponds to a probability of developing CJD spontaneously according to the German Health Authority. In the following a simplified summary of the classification system is given.

Parameter 1 (*origin and feeding of animals*): The factor describes the probability that animals have been infected. A factor 1 is applied for animals from unknown origin, taken into account the worst scenario that all animals from this origin are BSE-infected. A factor 3 would be applied to products derived from cattle from countries with a BSE prevalence of 10^{-3}-10^{-4} (e.g. Ireland). Factor 6 is applied for countries: without BSE cases, and with obligation for announcing BSE cases since 1992, and with import restrictions for cattle from UK since 1990. Herds are called closed if there is either no exchange with animals from other herds or exchange only within closed herds. For such animals a maximum factor of 8 can be applied if there is documented evidence: (1) that at least for 6 years, there was no BSE case reported; (2) there was no feeding with insufficiently heated bone meal; (3) no female animal from other herds not fulfilling these criteria was included.

Parameter 2 (*raw material*): This parameter takes into account that the amount of prions in infected animals varies considerably within different tissues. Brain, spinal bone marrow and eye belong to class 0 as these tissues contain high concentrations of prions in infected animals. Class 5 contains e.g. liver, lung, pancreas. Tissues bearing the least risk of infection belonging to class 8, include heart, skeletal muscles, bone and cartilage.

Parameter 3 (*inactivation*): This classification considers the reduction of infectivity of single processing steps during production. The German model requires validation for all production steps which are used for the calculation. If data from the literature are used instead of experimental validation, only $\frac{3}{4}$ of the factor described in the literature can be applied.

Parameter 4 (*raw material per daily dose*): The classification is based on the amount of raw material used for the production of the maximum daily dose. Class 0 corresponds to < 1 kg and > 100 g raw material per daily dose, class 1 corresponds to < 100 g and > 10 g, class 2 < 10 g and > 1 g, etc.

Parameter 5 (*number of daily doses*): The highest number of days has to assumed on which the product is used: class 0 stands for long-tem use, i.e. 100-365 daily doses per year, class 1 for 10-99 daily doses per year, while class 2 corresponds to 1-9 daily doses per year.

Parameter 6 (*method of application*): Direct application into the central nervous system is considered to carry the highest risk of infection. Therefore, this application is classified as class 0. Application into blood stream corresponds to factor 1, while other parental applications are classified as factor 2 applications. Application onto intact skin corresponds to factor 7.

2.2. *Measuring of residual proteins in Bio-Oss®*

Each batch of Bio-Oss® is routinely tested for residual proteins by two independent methods: determination of

total protein, and determination of 4-hydroxyprolin. For measuring residual proteins 5 g Bio-Oss® is dissolved in hydrochloric acid, neutralized by using NaOH and finally the volume is adjusted to 50 ml with destilled water. This solution is assayed for proteins.

The method described by Lowry and coworkers is the most widely used test for determination of protein concentration [12]. The method is based on the reaction of folin and a phenol reagent with functional amino acid groups contained throughout the protein polypeptide chains. The reagent's reaction with its substrate in solution leads to an intense blue colour, that is proportional to the protein concentration. When bovine serum albumin (BSA) is used as a standard the detection limit is 135 ppm.

The photometric method of Bergman and Loxley for the determination of 4-hydroxyprolin as a characteristic component of collagen is based on the oxidation of hydroxyprolin to a compound related to pyrrole and the subsequent condensation of this intermediate compound with Ehrlich's reagent to form a red dye [13]. The detection limit of the method lies at 22 ppm.

2.3. BSE inactivation study

The purpose of these experiments was to determine the inactivation of prions due to the alkaline treatment during the preparation of Bio-Oss®. The inactivation was monitored by the disappearance of the disease-specific form of the prion protein (PrPSc). This marker is strongly correlated with infectivity [18]. The inactivation is monitored by the change of the protease-resistant, disease-specific form of the prion protein to a protease-sensitive inactive form [20]. Using a validated Western blotting procedure (Prionics Check [7]; Report of the evaluation of the European Commission of BSE tests, available at: http://europa.eu.int/comm/dg24/health/bse/bse12_en.html). PrPSc is detected as a protease-resistant fragment of 27-30 kDa (PrP 27–30). Under the same conditions PrPC is completely digested by proteases therefore allowing its distinction from PrPSc.

BSE brain homogenates were prepared by Prionics from confirmed Swiss cases of BSE for analysis by Western blotting (Prionics Check). Briefly, a 10% BSE homogenate (w/v) was prepared by homogenizing BSE brain tissue in the appropriate volume Prionics Check homogenization buffer using an OMNI GLH homogenizer set at 20 000 rpm. As negative controls homogenates from confirmed negative animals of the Swiss BSE surveillance program were used. To determine the prion inactivation capacity of the alkaline treatment used in the process of manufacturing Bio-Oss® the following bioassay was performed. Mouse scrapie brain homogenate is incubated for 2 h at 20°C with either the alkaline solution as it is used for the alkaline treatment of Bio-Oss® or with PBS as a control. The alkaline treated homogenates were neutralized using HCl and dialyzed for 3×1 h against 3×1 l 100 mM Na phosphate pH 7.4. As a further control non-treated homogenates were used. The dialyzed homogenates were then analyzed directly by the Prionics check procedure (i.e. proteinase K digestion followed by Western blotting) as described in Schaller et al. [7].

As an alternative, proteins were precipitated after the proteinase K digestion and then resuspended $20\times$ more concentrated for Western blot analysis. To validate this procedure, BSE-homogenate was diluted into normal brain homogenate, then treated with proteinase K and analyzed by Western blotting. These control experiments assured that PrP^{Sc} diluted into normal brain homogenate could indeed be concentrated and detected by Western blotting.

3. Results

3.1. Risk assessment according to Model of the German Health Authority

For Bio-Oss® a sum of classification factors of in the best case 38.7 in the worst case 26 was calculated. For Osteograf/N the risk assessment revealed a factors of either 26.3 or 24.5 (Table 1). Worst-case calculation

Table 1
Risk assessment for Bio-Oss® and Osteograf/N according to the German Health Authority Model[a]

	Bio-Oss® worst case scenario	Bio-Oss® best case scenario	Osteograf/N worst case scenario	Osteograf/N best case scenario
Origin	7	7	7	7
Starting material	8	8	8	8
Method of prion inactivation	7	19.7	5.5	7.3
Starting material per daily dose	0	0	0	0
Number of daily doses	2	2	2	2
Site of application	2	2	2	2
	26.0	38.7	24.5	26.3

[a]The best-case scenario was calculated according to the highest values described in the literature. The worst case scenario was calculated by strictly following the German Health Authority and wherever possible by experimental evidence.

Table 2
Risk assessment of the manufacturing process of Bio-Oss® according to the German Health Authority Model

Processing step	Factor from literature	Factor applicable for risk assessment ($\frac{3}{4}$ of factor from literature)
Organic treatment for fat extraction	0[a] or 7.4	0* or 5.6
Heat treatment > 300°C	5.3–7.3	4–5.5
Alkaline treatment	5	3 (validated)
Sum	10.3–19.7	7–13.6

[a] As the organic solvent used for the production of Bio-Oss® is not exactly the same as the one used in the prion inactivation studies, this purification step is not considered in the worst case scenario.

corresponds strictly to the requirements of the German model, i.e. using only $\frac{3}{4}$ of factors for inactivation if referenced from literature only.

Parameter 1 (*origin and feeding of animals*): Bio-Oss® is produced from U.S. bovine bone only. USA is a class 7 country. Animals, from which bones are used for manufacturing Bio-Oss®, must be examined by officials of the United States Department of Agriculture (USDA). Contract vendors are USDA-registered slaughterhouses which work under direct supervision of USDA inspectors. Each individual is examined by USDA inspectors. These examinations include inspections for visible manifestations of disease as well as post-mortem examinations. According to the new German model of 1996 a factor 8 can only be applied for animals from closed herds [6]. Based on the information given in the recent publication a factor 7 is applied for Osteograf/N, as well [11].

Parameter 2 (*raw material*): Both bone substitutes are prepared from bones which are classified as class 8 materials corresponding to the highest safety class. The bones used for Bio-Oss® have to fulfil strict quality requirements regarding freshness as well maturity of bone structure. Controls at the vendor during slaughtering as well as 100% incoming controls at the manufacturing site are performed.

Parameter 3 (*inactivation*): Treatment of the starting material (bovine bone) with strong alkaline solution is considered a very effective step in eliminating the infectivity of prions. Treatment of laboratory strains of cattle or hamster prions with 1–2 M NaOH for 30–120 min resulted in an at least 100 000 fold reduction of infectivity [14,15]. Under the same conditions, BSE prions were almost completely inactivated [14]. Another step involves the treatment of the material with organic solvents leading to the extraction of fat. Such a treatment will destroy the three-dimensional structure of proteins due to the hydrophobic nature of organic solvents. Treatment of prions with organic solvents such as 0.1% phenolic solvent/0.05% hexylene glycol for 16 h lead to a complete loss of infectivity (i.e. a more than $10^{7.4}$ fold reduction of infectivity [16]. Treatment of hamster prions (which are generally assumed to be more stable than bovine prions) at 160 or 360°C for 1 h resulted in a reduction of the infectivity by a factor $10^{5.3}$ and $10^{7.3}$, respectively [17]. Since Bio-Oss® is heated to > 300°C for more than 10 h an inactivation factor of $10^{5.3}$–$10^{7.3}$ should be achieved.

According to the literature the combination of all inactivation factors leads to an estimate of a $10^{10.3}$ (in the worst case) to $10^{19.7}$ (in the best case) fold reduction in infectivity during the production process of Bio-Oss® (Table 2). Since the organic solvent used in the production of Bio-Oss® is not exactly the same as that used in the prion inactivation studies, it has to be considered that this step might not be as effective. Therefore, for the worst-case scenario, the organic solvent extraction step was not considered. In a best case assumption, i.e. consideration of maximum inactivation factors, a factor 19.7 could be applied for Bio-Oss®. However, the German authorities require that inactivation factors referenced from the literature, are valued only for $\frac{3}{4}$ of the factor. Therefore, for Bio-Oss® a factor of 4 to 5.5 for the heat treatment, a factor of 5.6 for the fat extraction plus a factor 3 for the validated alkaline treatment step (see below) can be applied.

Osteograf/N is treated with heat in excess of 1000°. Mathematical extrapolation of the scientific data based on assumptions is not possible according to the German model [5,6]. Therefore, an inactivation factor of 5.5 ($\frac{3}{4}$ of 7.3, as described for heat in the literature [17]) is applied.

Parameter 4 (*raw material per daily dose*): The maximum size of Bio-Oss® is a 2 g vial. For large augmentations such as sinus floor elevations or multiple defect filling several vials may be used in one patient during surgery. For the worst-case calculation a maximum single dose of 10 g is assumed. For the production of 10 g Bio-Oss® about 105 g fresh raw bone material is needed. This corresponds to class 0 (< 1 kg and > 100 g).

For Osteograf/N one batch raw bone was described as consisting of 1000 lb, i.e. 453 kg [11]. 6000 vials of 1 g are prepared from one batch which means that 755 g fresh

bone are needed for a maximum daily dose of 10 g. This also corresponds to class 0.

Parameter 5 (*number of daily doses*): For bone substitutes a frequency of application of less than 10 per year is reasonable. For both products a classification factor 2 can be applied.

Parameter 6 (*method of application*): Application into open wounds as bone defects is classified as a factor 2 application.

3.2. Measuring residual protein content of Bio-Oss®

The manufacturing process of Osteograf/N involves temperatures in excess of 1000°C. It is extremely unlikely that the final product contains protein as all known proteins are ashed at these temperatures. Therefore, we have not measured the protein content of Osteograf/N. However, Bio-Oss® was tested for residual proteins by two independent and validated methods: determination total protein according to Lowry et al. [12], and determination of 4-hydroxyprolin by the method of Bergman and Loxley [13]. Based on the results of more than 900 examined batches no proteins could be detected (data not shown) and Bio-Oss® can therefore be concluded to be protein free.

3.3. BSE inactivation measurement

The purpose was to determine the inactivation of prions during the preparation of Bio-Oss®. To achieve this goal, the initial step for the preparation (alkaline treatment) was reproduced in the laboratory with BSE prions. The inactivation was judged by the disappearance of the disease-specific form of the prion protein (named PrP^{Sc}). This biochemical marker is strongly correlated with infectivity as the infectious particle seems to be composed in part, if not entirely, of PrPSc [18,19]. The inactivation was monitored by the change of the protease-resistant, disease-specific form of the prion protein to a protease-sensitive form which correlates with inactivation [20]. Using a validated Western blotting procedure (Prionics Check; [7]) PrP^{Sc} is detected as a protease-resistant fragment of 27–30 kDa (PrP 27-30) while under the same conditions PrPC is completely digested by proteases thus allowing its distinction from PrP^{Sc}.

Immunological detection of proteins after electrophoretic separation on SDS-polyacrylamide gels has become a standard technique in many research laboratories (Western blot procedure) and has been applied to the detection of prion proteins for a long time [21]. Posttranslational modifications of PrP lead to a characteristic pattern of three major bands for PrP (from 25 to 35 kDa) which differ in their N-linked glycosylation. The protease-resistant fragment of PrP^{Sc} lacks about 60 amino acids at the N-terminus leading to a shift in the electrophoretic migration pattern of about 5–6 kDa. The protease-resistant fragment PrP 27–30 is very characteristic for the presence of disease-specific PrP and is never observed with normal PrPC.

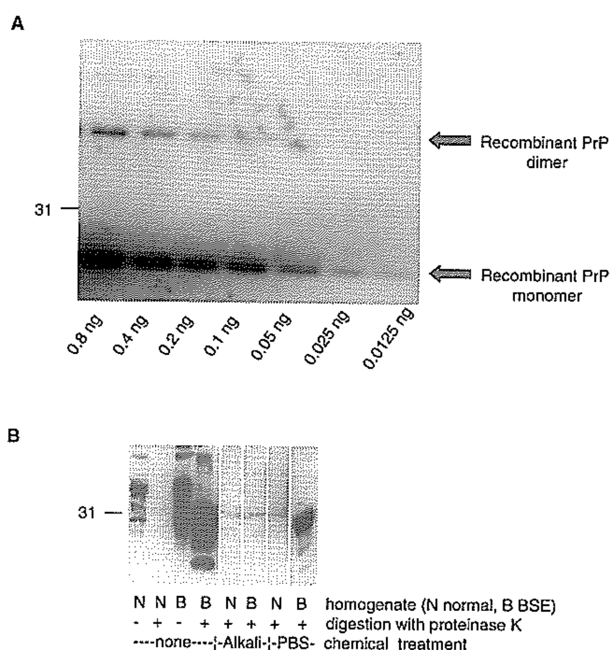

Fig. 1. Detection of PrP. (A) Sensitivity of Western blot tested with defined amounts of recombinant PrP. Serial dilutions of highly purified recombinant PrP were separated by SDS PAGE and blotted to PVDF membrane. PrP was detected using the monoclonal anti-PrP antibody 6H4 followed by a goat-anti-mouse-IgG antibody coupled to alkaline phosphatase (AP). AP activity was detected by chemiluminescence. (B) Analysis of normal and BSE homogenates before and after alkaline treatment. The first 4 lanes represent the standard analysis done for bovine homogenates with Prionics Check. The PrP in normal homogenates is completely protease-sensitive while PrP^{Sc} in BSE homogenates leads to a reduction in molecular weight. The second 4 lanes represent normal or BSE homogenate treated either with alkali or PBS followed by protease digestion. After the chemical treatment with alkali, PrP^{Sc} in the BSE homogenate has become completely protease sensitive, while a BSE homogenate treated in parallel with PBS still shows the characteristic protease-resistant fragment of the prion protein.

Different amounts of purified recombinant bovine PrP were analyzed under the conditions specified in the Prionics Check procedure (Fig. 1A). Concentrations down to 12.5 pg PrP could be detected. The higher bands observed in the lanes with high concentrations of PrP represent non-covalent, SDS-resistant aggregates of PrP. A dimer of recombinant PrP is indicated. Note that in samples containing PrP^{Sc} also a higher band probably corresponding to a dimer of PrP 27–30 can be observed.

Fig. 1B shows the effect of the alkaline treatment on PrP^{Sc}. On the left side, the controls with 10% brain homogenate show that PrP in normal brain homogenates is protease sensitive while PrP^{Sc} in BSE homogenate is cut partially yielding a major protease-resistant fragment of 27–30 kDa. The alkaline treated

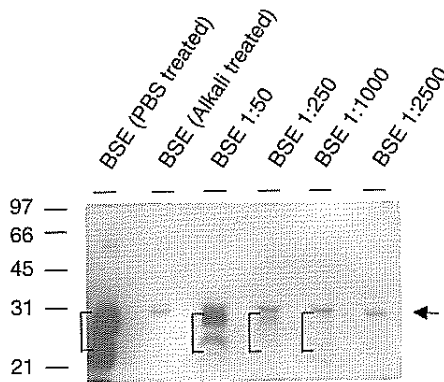

Fig. 2. Limit of detection for PrP^{Sc}. BSE brain homogenate was treated either with PBS or alkali and then incubated with proteinase K. To increase the sensitivity, samples were concentrated by protein precipitation followed by Western blot analysis. As a control dilutions of BSE homogenate in normal brain homogenate were processed in parallel to determine the sensitivity. PrP 27–30, the protease resistant fragment of PrP^{Sc} is denoted by brackets. The band at 31 kDa (arrow) indicates proteinase K.

samples of normal or BSE homogenates did not show any PrP immunoreactivity while in PBS treated BSE homogenate, the characteristic PrP^{Sc}-fragment was detected.

To assess the sensitivity of the experiments, dilutions of BSE homogenate in normal brain homogenate were analyzed (Fig. 2). The sensitivity was increased by enrichment of the samples using non-specific protein precipitation. At a dilution of 1:250 a clear PrP 27–20 signal can be detected, while at 1:1000 only a very faint signal is seen. No signal is seen at higher dilutions. The limit of detection for PrP^{Sc} left after the alkaline treatment is therefore at a 1000x lower concentration than in the original BSE homogenate.

In all the experiments positive and negative controls performed as expected (data not shown). Dilutions of positive controls were reliably detected while negative control samples were consistently negative.

The titer of mouse prions in brain is usually in the range of 10^7–10^8 infectious units (IU) per gram of tissue. For BSE, the titer of individual animals may vary dramatically ranging from 10^4 to 10^7 IU/g tissue leading to the highest possible titer of 10^6 IU/ml. It is possible that the titers in the actual homogenates used in the experiments might have been lower but this would mean that even lower amounts of infectivity would have been detected than currently assumed. To calculate the amount of IU equivalents detected on the Western blots the highest titers possible for the original homogenates, i.e. 10^6 IU/ml was assumed. Five microliters of brain homogenate were loaded per lane. The PrP 27–30 signal of a 10% BSE homogenate therefore corresponds to 5×10^3 IU. By applying a concentration step, we detected a (250–1000)-fold dilution of the BSE homogenate in normal brain homogenate. It can therefore be deduced that about 5–20 IU can be detected in this assay.

4. Discussion

Bovine bone substitutes are widely used for treating osseous defects. Due to their bovine origin risks of transmitting bovine spongiform encephalopathy to humans have been discussed [22–24]. As some of these materials are routinely and successfully used in surgical dentistry and orthopedic surgery careful risk assessment has to be done. The bone substitutes discussed here are manufactured from US cows only. As the United States of America is considered to be a country without BSE-cases it makes it unlikely that the starting material for the manufacturing of Bio-Oss® or Osteograf /N contains prions. However, it recently became questionable whether the US can still be considered as a BSE-free country (see the following web page of the European Union for details: http://europa.eu.int/com/dgs/health_consumer/library/press/press66_en.html). Furthermore, many biomaterial scientists, dental and orthopaedic surgeons getting more concerned about the bio-safety of biomaterials from bovine origin. Therefore, there is an increasing interest in the analysis of the risk of transmitting BSE through grafting materials derived from bovine bone.

Risk analysis can either be based on theoretical assessments or experimental evidence.

Here we present a comparative study on two bone substitutes (Bio-Oss® and Osteograf/N) which is based on theoretical values. Furthermore, for one of these materials, i.e. Bio-Oss®, the prion inactivation capacity of one of the production steps was experimentally evaluated. Theoretical and experimental data indicate that the use of these materials do not carry a measurable risk of transmitting BSE to patients.

The theoretical risk assessment has been performed according to a model proposed by the German health authority [5,6]. This has been done before for Osteograf/N based on a model published in 1994 [5]. However, recent updates of the guidelines based on new research results required re-evaluation of the published data. The new values for Osteograf/N are between 24.5 and 26.3, whereas in the former publication a factor of 30 was calculated. For Bio-Oss® values between 26 and 38.7 were calculated. As the sum of factors must attain a value of at least 20 points for the safety criteria to be considered fulfilled both products are safe. Sogal and Tofe set the risk of transmitting BSE to humans through bone substitutes vividly in relation to the risk of being killed through lightning or tornadoes [11]. They concluded that there is an orders of magnitude higher risk associated with such natural events.

For this theoretical evaluation we used a best- and a worst-case scenario for both of these products. In

a worst-case assumption the German model was strictly followed and only $\frac{3}{4}$ of the factors for inactivation referenced from literature was applied. However, data from literature also suggest that higher inactivation factors can be considered [25–27]. Therefore, also a best-case assumption was made. In none of both cases a species barrier was calculated since there are no scientific data allowing conclusion on this item.

Since most of the infectious agents contain proteins the estimation of protein content in a product may be an important aspect for the safety assessment. Many different methods are available for determining protein concentration. One has to be very careful to choose the proper method for each material. Otherwise, proper estimation of the protein content is impossible [28]. Therefore, we used validated methods for the estimation of protein content as described in the material section. Based on the results of the determination of total amount of protein and 4-hydroxyprolin there is no evidence for the presence of protein in Bio-Oss®.

The alkaline treatment completely abolished detectable PrP^{Sc} in the experiments. The minimal reduction factor for inactivation is given by the signal detected in dilutions of the starting material, i.e. 10^{-3} for the experiments presented here but it could well be higher as it has been shown for alkaline treatments of prions by other research groups. Treatment of laboratory strains of cattle or hamster prions with 1–2 M NaOH for 30–120 min resulted in an at least 100 000 × fold reduction of infectivity [14,15]. BSE prions were almost completely inactivated [14].

The alkaline treatment used for the production of Bio-Oss® is around pH 13, therefore representing even stronger conditions than those used above by Taylor et al. [14,15]. In accordance with these previous studies, the results (based on the detection of PrP^{Sc} by Western blotting) have indicated complete inactivation of PrP^{Sc} (i.e. conversion from protease-resistant to protease-sensitive PrP). The limitations on the biochemical detection of PrP^{Sc} impose the reduction factor of 10^{-3}.

The reproducibility of the Prionics Check procedure has been evaluated by the European Commission (Preliminary Report concerning the evaluation of tests for the diagnosis of Transmissible Spongiform Encephalopathy in Bovines (8 July 1999) as well as the Swiss Veterinary Authorities and therefore represents a recognized method for analysis of PrP^{Sc} and detection of prion-infected animals [7].

The safety of a medical product does not only mean the absence of side effects or biological safety. Medical efficacy and reliability are of at least similar importance in the risk to benefit analysis. With bone substitutes the osteoconductivity is of uttermost importance in order to ensure good clinical prognosis. Bovine bone substitutes with proven record of medical efficacy and with a validated risk analysis can therefore be considered safe and can be recommended for clinical use.

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