Experimental bovine spongiform encephalopathy infection in rabbits

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Abstract. The aim of our work was to determine the sensitivity of rabbits to the C-BSE pathogen in the first passage, to obtain a laboratory model on rabbits to study the C-BSE pathogen. The results of the experiments showed that with intracerebral infection of rabbits with the causative agent of classical spongiform encephalopathy of cows (C-BSE) they are 100% sensitive to the pathogen with an incubation period of 570 to 1842 days. The disease proceeds with the development of a clinical picture typical of prion diseases, the development of pathomorphological changes in the central nervous system in the form of vacuolization of neurons and neuropiles in various parts of the brain and massive deposition of prion protein PrPSc both in brain tissues and in peripheral lymphoid tissue (spleen). The laboratory model on rabbits is a highly sensitive model for studying the prion of bovine spongiform encephalopathy (C-BSE) and probably the causative agent of v-CJD.

1 Introduction

It is now generally accepted that prion diseases in animals and humans are absolutely fatal neurodegenerative infections caused by an aberrant form of the cellular prion protein PrPSc resistant to proteinase K [1]. Although a wide range of mammals are susceptible to prions, prion diseases have been reported sporadically. However, sheep and goat scrapie, transmissible mink encephalopathy, bovine spongiform encephalopathy, and chronic deer wasting can occur as epizootics and pose serious economic and social problems.

Experiments on laboratory animals traditionally play an important role in elucidating the etiology and understanding the pathogenesis of infectious diseases. In search of a laboratory model cheaper than monkeys for studying the pathogens of Kuru and Jakob-Creutzfeldt, a number of laboratory animals, including rabbits, were tested for sensitivity to them. In parallel, sheep scrapie prions and mink encephalopathy were used in the experiment. Positive results were obtained with the causative agents of scrapie and mink encephalopathy, however, rabbits and guinea pigs did not show clinical signs of the disease to the tested pathogens during the three years of observation [2]. Similar results were also obtained by other researchers with intracerebral infection of rabbits and guinea pigs with...
the causative agent of scrapie, strain ME7 [3]. The bovine spongiform encephalopathy [BSE] epizootic of the 1980s due to exposure to the pathogen in the environment and ingestion of feed contaminated with meat and bone meal revealed a wide range of susceptible animals, but cases of horses, pigs, dogs and rabbits were not registered. The resistance of these animals to infection with pathogenic prions under experimental and epizootic conditions caused interest in establishing the causes of this phenomenon. The study of the nucleotide sequence in the rabbit prion protein gene showed 82–87% identity to other mammalian sequences, and the sequence of amino acid residues in PrP\(^C\) proteins was 88–93% identical, and the authors concluded that the resistance of rabbits to prion infection is associated with special differences in the region of the central core of their PrP\(^C\) [4]. Investigating the effect of seven substitutions in the prion protein gene in a mouse neuroblastoma cell line constantly producing the scrapie prion with rabbit amino acid residues, it was found that expressed rabbit PrP did not convert to PrP\(^{\text{Sc}}\) under the influence of four substitutions. The authors concluded that the critical amino acid residues that inhibit the conversion of PrP\(^C\) to PrP\(^{\text{Sc}}\) are located throughout the rabbit PrP\(^C\) sequence [5]. When 4 more substitutions were added, out of 11 only two substitutions N100Y and N109M suppressed PrP conversion [6]. The results of these experiments turned out to be contradictory and allow us to speak about the absence of a cooperative effect of effective substitutions and the presence of influence on the process of prion protein conversion and other factors.

A multidimensional heteronuclear magnetic resonance study of the effect of substitutions in the I214V and S173N nucleotides on the properties of the PrP\(^C\) prion protein showed that the substitutions lead to its structural changes, while the flexibility of the ordered β2-α2 loop and the surface charge distribution significantly affect its conformational stability [7-8].

The role of the β2-α2 loop of the rabbit prion protein and the tail of helix 3 in its stability was studied by computer simulation of molecular dynamics under various environmental conditions. In the experiments, we used the rabbit prion protein structures identified by X-ray diffraction analysis and nuclear magnetic resonance, registered in the Protein Data Bank (https://www.rcsb.org/). The results of molecular dynamics modeling showed that three α-helices of the wild rabbit prion protein are stable at neutral pH, while in the presence of I124 and S173N mutations, the prion protein converts to the β-state under these conditions. The stability of the prion protein is also maintained by salt bridges ASP201-ARY155 and ASP177-ARY163(O-T) [9-13].

In a comparative in vivo study of the effect of prion protein on the development of pathology in transgenic flies producing hamster, mouse, and rabbit PrP\(^C\), it was found that the transgenic fly model is sensitive to subtle differences in the sequences of hamster and mouse PrP\(^C\), which cause different pathology in flies after their conversion to PrP\(^{\text{Sc}}\). Rabbit PrP\(^C\) remains stable, is not subject to conversion, and, accordingly, does not cause pathology in transgenic flies [14, 15, 16].

It is known that proteins have a common property of aggregation into non-functional structures and this is a universal problem for all cell types, which is exacerbated by a high concentration of macromolecules in intracellular structures. This phenomenon was called the accumulation of molecules [17]. When studying the effect of aggregation conditions induced by the crowding agents ficoll 70 and dextran 70 on full-length recombinant human, bovine, and rabbit PrPs, it was found that these agents dramatically increase the formation of fibrils of the first two proteins and significantly inhibit fibrillation of the rabbit protein, stabilizing its initial state. The fibrils formed by the rabbit protein contained fewer β-sheet structures and more α-helices than the PrP fibrils of human and bovine proteins. Moreover, rabbit protein amyloid fibrils, in contrast to human and bovine protein amyloid fibrils, did not produce a proteinase K resistant 15-16 kD nucleus. The authors speculate that it is
therefore "unlikely that rabbit PrP will cause prion disease" [18-19]. This assumption is consistent with studies of the level of expression and distribution in rabbit tissues of the precursor of the laminin 37/67 receptor, a decrease in the level of which prolongs the incubation period of experimental prion infection. Very low levels of expression have been established in the tissues of the central nervous system of the rabbit, in contrast to animals sensitive to prions [20].

However, when an uninoculated normal rabbit brain homogenate and the same homogenates inoculated with different strains of murine and sheep scrapie prions, as well as bovine spongiform encephalopathy (BSE) and deer chronic wasting (CWD) were subjected to serial automated cyclic amplification with improper protein conversion (sa PMCA), rabbit PrP\(^c\) was found to be sensitive to conversion, since the prion protein of all inoculated strains generated protease K-resistant rabbit PrP\(^{res}\) (RaPrP\(^{res}\)) by the seventh round, while the non-seeded homogenate produced RaPrP\(^{res}\) by the thirteenth round [21]. The last, from the uninoculated homogenate, RaPrP\(^{res}\) was administered intracerebrally to three rabbits, one of which fell ill after 766 days with the development of incoordination and dullness. Western blot in his brain tissues revealed PrP\(^{Sc}\) with typical migration bands; histological analysis of brain tissues revealed vacuolization of the neuropil and cytoplasm of neurons, and immunohistochemistry revealed astrogliosis and the presence of PrP\(^{Sc}\). The resulting rabbit prion strain showed higher resistance to RA treatment and guanidine denaturation than the ME7 and RML strains of scrapie. At the second passage in rabbits, this strain caused disease in two out of 10 animals. Based on these experiments, the authors concluded that “rabbits can no longer be considered a prion-resistant species” [21]. Subsequently, the authors repeated the second passage of the new rabbit prion strain RaPrP\(^{res}\) on five rabbits. As a result, 100% incidence was registered with an average incubation period of 569.4 ± 12.4 days. The authors characterize the strain as weakly pathogenic [22].

A normal rabbit brain homogenate was seeded with C-BSE prions and subjected to several rounds of amplification by serial automated PMCA, resulting in a misfolded rabbit PrP\(^c\) and a proteinase K resistant BSE-RaPrP\(^{res}\), i.e. those. C-BSE prions adapted to rabbits. When they infect transgenic mice expressing bovine or human prion protein, they become ill and die. All of them showed sponge-like changes in the neuropil of the gray matter of various parts of the brain. Immunohistochemical method revealed the accumulation of prion protein both in the form of large-plaque and fine-grained deposits inside - and perineuronal. In general, the strain retained the pathobiological properties of the C-BSE strain [23].

Although it has been experimentally shown that rabbits are susceptible to prions, including the C-BSE prion, the analysis of the above works shows that in no experiment was direct infection of rabbits with the C-BSE prion, and the question of their sensitivity to prions in the first passage remains open, although this question is the most important from an epizootological point of view.

2 Materials and methods

Ethics Statement. All experiments with animals were carried out in strict accordance with the international standard GOST 33044-14 "Principles of Good Laboratory Practice" and the Law of the Russian Federation "On Veterinary Medicine" No. 4979-1 dated 14.05.1993. as amended on April 29, 2023

Animals. In the experiment, 6 two-month-old Soviet chinchilla rabbits were used: four rabbits were used for infection, two as controls, which were kept separately from the infected in another room, they were cared for by their own staff. The rabbits were kept in individual cages in a special room. All animals were fed the same feed pool. Bedding,
leftover food and down, as well as corpses at the end of the experiment after taking tissue samples, were burned in a cremation oven. Animals were monitored daily in the morning and evening for clinical signs and food intake.

Prion strain. We used a strain of the causative agent of classical spongiform encephalopathy (C-BSE), obtained from the UK in the form of brain tissues of a sick cow, which underwent two successive passages in our laboratory on calves. A positive diagnosis of the disease in both passages was confirmed by the histological method and by ELISA using commercial diagnostic kits from BioRad and IDEX. The material for infection was prepared in a class III microbiology room using a Heidolph homogenizer. The homogenate (10% w/v) was prepared in sterile buffered saline (free of Ca$^{++}$ and Mg$^{++}$), pH 7.4, and clarified by centrifugation (3000 rpm, 5 minutes). 0.2 ml of the homogenate was injected intracerebral into the left hemisphere under general anesthesia by intramuscular injection of telazol according to the instructions. The craniotomy was performed with a drill with a diameter of 0.2 mm, the homogenate was injected with a No. 29g needle with a limiter to a depth of 1 cm. Two control rabbits were similarly injected with 0.2 ml of buffered saline, on which a homogenate was prepared.

Pathological material was taken from experimental animals at the terminal stage, from control animals after their euthanasia under anesthesia during the death of the first and last experimental rabbits. The selected material was fixed in a 10% buffered formalin solution for 10 days, after which it was examined histologically and by immunohistochemistry as described previously [24].

3 Results

All rabbits intercerebrally injected with the C-BSE prion fell ill with the development of clinical signs typical of transmissible spongiform encephalopathies, which characterize severe CNS damage. The incubation period in animals relative to each other differed significantly and amounted to 570, 690, 1840 and 1852 days; the clinical stage of the disease before the natural death of the animals was 10,14,11 and 17 days, respectively. Clinical symptoms in all affected rabbits were similar. At the beginning, there was a decrease in food and manifestations of anxiety, an increased reaction to the staff, then there was a violation of coordination of movement and a pronounced ataxia developed, there was a decrease in mobility and reaction to staff and noise, they became lethargic and indifferent, and death occurred. On the seventh day of the disease, the rabbit that fell ill with the first developed paresis of the hind limbs, and the animal that fell ill with the third developed a loss of vision without visible anatomical changes in the eyes. Control rabbits remained healthy throughout their observation period.

Pathological anatomical autopsy showed no differences from control rabbits in infected animals, all visceral organs looked normal. Histopathological changes were detected in infected rabbits in the tissues of the central nervous system in the form of vacuolization of the neuropil and neurons typical of prion encephalopathies. Lesions were recorded in all parts of the brain with varying degrees of intensity: in the cerebellum, significant vacuolization and cell loss were noted both in the outer zone of the molecular layer and in the ganglionic and granular layers. Vacuolization of neurons was also established in the pyromedial layer of the cerebral cortex. When analyzing brain tissues for the presence of the prion protein PrP$^\text{Sc}$ by immunohistochemistry, its deposits were detected in the form of individual plaques and coarse grains, as well as a diffuse distribution in the form of fine grains or dust covering significant areas of the neuropil. The prion protein PrP$^\text{Sc}$ was also detected by immunohistochemistry in the tissues of the spleen of diseased rabbits, which indicates the damage of the C-BSE prion, in addition to the CNS, also to lymphoid tissues.
4 Discussion

Elucidation of the susceptibility of rabbits to the causative agents of prion infections is of considerable interest from the point of view of finding a convenient laboratory model for studying the properties of prions and the search for drugs against prion diseases, and from an epizootic point of view, since rabbits are included in the human food chain. It is important to know if rabbits can be reservoirs and vectors of prion infections, especially BSE. The results of this experiment show that rabbits can be used to address these issues. All rabbits used for infection became ill, but the incubation period between the first and last diseased animals differed by more than three times. Such a difference in the incubation periods may be due to the genetic factors of the animals used, which were not studied in the experiment. The detection of PrPSc prion protein in the spleen of diseased rabbits indicates damage to the peripheral lymphoid tissue of diseased animals.

5 Conclusion

Our experiments with direct infection of rabbits with the C-BSE prion showed that rabbits are sensitive to the pathogenic protein, although the disease develops after a long incubation period. Clinical and pathohistological changes in the CNS in rabbits caused by the C-BSE prion, which is adapted to these animal species, were studied. Thus, rabbits can also be a laboratory model for the study of other prion diseases.

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