

1 **Intracerebral transfection of anti-rabies virus antibodies is an effective**
2 **therapy for rabies**

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27 **Abstract**

28 Rabies is a zoonotic neurological disease with 100% lethality. Some of the rare human
29 patients who survived after multiple drug treatment have inherited severe sequelae. The
30 objective of this study was to investigate the action of the transfection of antibodies against
31 rabies in the central nervous system of mice as target therapy for rabies.

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33 **Author summary**

34 The present study showed that after 48 h of RABV inoculation, mice injected by the
35 intracerebral route with anti-RABV F(ab')₂ complexed with Bioporter® Protein Delivery
36 Reagent (Genlantis) as a transfection agent, showing a morbidity/mortality rate of 30% with a
37 minimum incubation period of seven days, while in the control group a significantly higher
38 (p<0.0198) 90% morbidity/mortality was reached in thirteen days after a maximum 5-day
39 incubation period, suggesting that the transfection of anti-RABV antibodies into the brain might
40 prevent or delay RABV dissemination in an early stage of rabies infection. For the first time, a
41 single compound was able to inhibit replication of the virus in the nervous system with high
42 efficiency. This result can provide important results for the planning of protocols to prevent the
43 fatal outcome of the disease in advanced stages. New studies focusing on the optimization of
44 intracellular antibody delivery may be one of the main bases for more effective anti-rabies
45 therapy.

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48 **Introduction**

49 Rabies is a zoonotic neurological disease with 100% lethality; some of the few human
50 patients who survived after a multi-drug treatment developed severe motor impairment due to
51 ischaemic encephalopathy followed by necrosis of the hippocampus, cerebellum and cortex [1-
52 2]. The use of immunomodulators and antivirals has not been shown to be effective in inhibiting
53 the progression of the disease when tested in mice and humans [3-4]. Circa 59,000 human
54 deaths occur worldwide yearly due to rabies, and although it is preventable with pre and post-
55 exposure prophylaxis, the logistics and costs involved in rabies treatments are a limiting factor
56 to saving lives [5].

57 Rabies lyssavirus RABV (*Mononegavirales: Rhabdoviridae: Lyssavirus*) is a
58 neurotropic virus with a circa 11 Kb negative-sense single-stranded RNA as a genome that
59 codes for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), envelope glycoprotein
60 (G) and the RNA-dependent RNA-polymerase L protein [6], and it is most often transmitted
61 amongst mammals via saliva after an initial local replication in muscle cells that follows to the
62 central nervous system (CNS) via axons [7]. Within a variable period of time after infection,
63 signs of hyperactivity, hypersalivation and hydrophobia are detectable. The virus causes enough
64 damage to the brain in a few days that the infection invariably leads to coma and death by
65 cardio-respiratory arrest [8].

66 Here, we show that the use of intracerebral transfection of anti-RABV antibodies to treat
67 mice inoculated with RABV reduces mortality and extends the incubation period of rabies.

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70 **Results**

71 Probing the transfection to mouse brains with Bioporter agent complexed with an FITC-
72 antibody control protein (Genlantis) after intracerebral inoculations in mice showed fluorescent
73 foci at 4 and 6-hours post-injection in brain slices obtained in a cryomicrotome (Fig 1),
74 evidencing the efficacy of the protein transfection to mouse brains. However, the fluorescence
75 technique performed with microscopic slide tissue fragments showed absence of fluorescent
76 foci using equine anti-IgG conjugate for the mice inoculated with the F(ab')₂ anti-RABV
77 Bioporter complex at concentrations of 50 and 250 µg/mL and Bioporter, for post-inoculation
78 periods of 4 and 6 h (Unpublished data).

79 Mice which received anti-RABV F(ab')₂ in conjunction with Hepes 48 h.p.i., the mice
80 in the control group had onset of rabies symptoms at 5 days post injection. About 90% of the
81 mice showed symptoms of 2 to 3 days, followed by death, with only one mouse showing no
82 symptoms throughout the experiment, resulting in 90% mortality. Only one mouse presented
83 symptoms late, at 9 days p.i., consolidating survival of 10%. The clinical signs observed were
84 anorexia, piloerection, arching of the back, and limb paralysis before death.

85 On the other hand, the morbidity/mortality rate in the group treated with Bioporter plus
86 anti-RABV F(ab')₂ was as low as 30% with a minimum incubation period of seven days,
87 resulting in a significant difference ($p < 0.0198$) when compared to the control group (Fig 2).

88 Bioporter alone had no significant action on RABV, as morbidity/mortality rates of 50
89 and 80% were found for mice treated with only Bioporter or Hepes 20 mM pH 7.4 solution,
90 respectively, after 48 h of RABV inoculation ($p = 0.3498$), indicating that the reduced
91 morbidity/mortality rate in mice treated with anti-RABV F(ab')₂ transfected with Bioporter was
92 due to a specific intracellular neutralization effect (Unpublished data).

93 All dead animals were positive for direct immunofluorescence for RABV, with no
94 difference in fluorescence intensity between groups. Thirty days after viral inoculation, all

95 surviving mice were euthanized and negative by direct immunofluorescence and PCR for
96 RABV.

97 In summary, these results show that the transfection of anti-RABV antibodies into the
98 brain might prevent or delay RABV dissemination in an early stage of rabies infection.

99

100 **Discussion**

101 In this study, the IFD technique used with equine anti-IgG conjugate to detect a
102 transfection of anti-RABV F(ab')₂ through the Bioporter reagent performed 4-6 h after its
103 intracerebral injection showed no fluorescence, which may indicate that (a) transfection of anti-
104 RABV F(ab')₂ occurred with low efficiency, (b) its intracytoplasmic dispersion avoids large
105 clusters of anti-RABV F(ab')₂ accumulators, in this way the fluorescence of the conjugate anti-
106 IgG antibody is inhibited by fluorescence microscopy if it is associated with anti-RABV F(ab')₂
107 and (c), the result may be related to lack of affinity of the equine anti-IgG conjugate by the
108 fragmentation of IgG- RABV.

109 If the latter is the case, the absence of fluorescence should result from the purification
110 of anti-RABV F(ab')₂ by the enzymatic digestion of pepsin, which produces two F(ab')₂
111 fragments bound by disulfide bond, which reduces its molecular weight from 160 kDa IgG to
112 90 to 100 kDa, eliminating from the molecule the Fc fraction responsible for complement
113 activation by the classical route.

114 Interestingly, in the work done by Weill, among the three antibodies transfected in vitro
115 by the Pulsin reagent, the only one that did not demonstrate the expected signaling was the
116 Anti-mouse IgG antibody, since a secondary antibody rapidly exuded from the cytoplasm when
117 the cells were treated with digitonin (lipid solubilizer) revealing that it was not bound to any

118 target, while the primary antibodies remained within the cytoplasm for 15 minutes. This result
119 helps to support hypothesis (b) in which the dispersion of anti-RABV F(ab')₂ in the cytoplasm,
120 implies non-visualization of its location by signaling by the equine anti-IgG conjugate if it is
121 associated [9].

122 A fluorescence microscopy depends on detection of the fluorophore above its effective
123 detection limit. And, eventually, false negatives may occur when attempting to study the
124 dispersion of fluorophore labeled molecules, so that a low level of fluorescence could reflect
125 the absence in that tissue area or a high degree of dispersion would decrease the fluorescence
126 to the point of not being distinguished of the autofluorescence of the tissue attached to the
127 slide[10]. However, the abundance of FITC-control Bioporter, the product of transfection,
128 enabled confirmation of delivery by the reagent, even if no specific target was found
129 intracellularly.

130 Since the efficacy of post-exposure treatment decreases progressively when initiated
131 late, antibody performance is still significant if treatment is performed in the first 24 h. At this
132 early stage, treatment with antibodies performed directly in the CNS may still prevent or delay
133 the spread to the rest of the brain. However, in later stages, no protection is effective because
134 the virus has spread to larger parts of the brain [11].

135 However, inversely, our treatment performed 48 hours later with the Bioporter reagent,
136 70% of the mice in the treated group were healthy after viral infection, suggesting intense
137 inhibition of the virus by anti-RABV F(ab')₂ and , in addition, there was an increase in survival
138 of 48 h for the rest of the mice that died. The anti-RABV F(ab')₂ was transfected using a single
139 intracerebral inoculation with optimal result. These results are consistent with previous
140 observations showing inhibition of viral activity in N2A cell culture inoculated with isolates
141 DOG-IP3629/11 [12]. Therefore, transfection of anti-RABV F(ab')₂ *in vivo*, demonstrated in

142 this study could act in other variants with the same result confirmed for (IP3629/11 dog),
143 especially in cases of human rabies.

144 The possibility of the Bioporter reagent contributing to the antiviral action together with
145 the anti-RABV F(ab')₂ required further investigation. When this possibility was tested, the
146 Bioporter reagent was inoculated into mice in the CNS without addition of anti-RABV F(ab')₂.
147 This approach led to 50% and 20% survival between the Bioporter and Hepes salts respectively,
148 without significant difference (p=0,3498), demonstrating that the inhibitory effect of treatment
149 was due only neutralizing action of anti-RABV F(ab')₂.

150 Infections in the CNS are contained by the action of several immune effectors such as
151 antibodies, cytotoxic T-cells and soluble factors that are involved in generation and control of
152 the immune response as type-1 IFNs. Consequently, after brain infection by a pathogen, MHC
153 II expression is surprisingly upregulated by approximately 90% by glial cells, including in
154 diffuse areas distal to viral infection [13]. In murine brain infection demonstrates the prolonged
155 activation of microglia associated with the continued presence of long-lasting memory T cells
156 in the brain. Thus, it is clear that this small number of long-term memory T-cells may advance
157 control of reinfection or reactivation of pathogens in the CNS by directing innate immune cells
158 as microglia [14].

159 In some reports, the serological status of the patients shows that the production of
160 antibodies plays a fundamental role in viral clearance. Prior to the Milwaukee Protocol, few
161 human cases of rabies survival received post-exposure prophylaxis only with administration of
162 the vaccine [15, 16, 17, 18]. Complete recovery or with sequels in rabies patients is limited to
163 a few cases in the literature linked to the history of immunization with the combination of
164 vaccines and passive immunization of antibodies at the onset of symptoms [19, 20, 21]. Studies
165 with B cell-deficient mice, which underwent CNS virus inhibition after peripheral

166 administration of RABV-specific antibodies at 5 d.p.i, support these results. Because, passively
167 administered antibodies gain access through the BBB, conferring therapeutic antiviral effects
168 on the CNS. This indicates that neutralizing antibodies may be able to cross the blood-brain
169 barrier, representing an increase in the patient's expected life expectancy until immunotherapy
170 can establish the response against the virus [22, 23].

171 Anti-RABV antibody transfection as shown in this report is a candidate new tool for the
172 treatment of rabies when the disease has already manifested, such as in cases in which no post-
173 exposure prophylaxis was applied or in cases the prophylaxis failed. We have added a new tool
174 for manipulation in the against rabies, by inhibiting replication and viral synthesis in neural
175 cells without affecting neurotransmitters, using a single intracerebral inoculation with optimal
176 results.

177

178 **Materials and Methods**

179

180 **Ethics.** This experiment was approved by the Ethics Committee on Animal Use (CEUA) of
181 the School of Veterinary Medicine and Animal Science - University of São Paulo (FMVZ -
182 USP), under Protocol no. 9658071016. All mice were used for the experiments; prior to any
183 procedure, mice were anesthetized with isofluoran.

184 **Transfection test with Bioporter® protein delivery reagent *in vivo*.** First, in order to assess
185 the effectivity of protein delivery to mice brains, a total of 40 µl of FITC-antibody control
186 protein (Gelantis) was complexed with the Bioporter® Protein Delivery Reagent (Genlantis)
187 per the manufacturer's instructions and was injected by the intracerebral route in two mice, and
188 the CNS of each mouse was collected at 4 and 6 hours post-injection. Ten-µm sections were

189 obtained in a LEICA CM 1860 UV microtome, fixed on glass slides with -20°C acetone for 2
190 hours and observed for fluorescence with an OLYMPUS ® BX53 epifluorescence microscope.

191 **Transfection test with anti-RABV F(ab')₂ *in vivo*.** Next, forty-two female 21-day-old CH3
192 ROCKEFELLER mice were inoculated with RABV strain IP3629/11-AgV2 isolated from a
193 dog in Brazil on mouse central nervous system (CNS) with a titer of 10^{3.8} DL₅₀/ μL kindly
194 provided by the Pasteur Institute, Brazil. After 48 h, mice were intracerebrally injected with 40
195 μL of either Bioporter resuspended in Hepes 20 mM pH 7.4 containing anti-RABV F(ab')₂
196 (treated group, n=10 mice) or Hepes 20 mM pH 7.4 containing anti-RABV F(ab')₂ (control
197 group, n=10 mice), both groups with 0.17 UI (250 μg) of anti-RABV F(ab')₂ as a final dose.

198 **Evaluation of the action of the transfection agent alone on RABV.** To assess whether the
199 Bioporter transfection agent alone had any effects on RABV, the aforementioned experiment
200 was repeated, but the (test group, n=10 mice) was injected with 40 μL of Bioporter in 400 μl
201 Hepes 20 mM pH 7.4, and the (control group, n=10 mice), was injected with 40 μL of Hepes
202 20 mM pH 7.4 solution after 48 h of RABV inoculation.

203 **Antibodies.** Anti-rabies hyperimmune serum was kindly provided by FUNED (Fundação
204 Ezequiel Dias), Brazil, containing enzymatic-digestion purified equine IgG Fab fragment
205 against the PV strain of RABV (200UI/mL).

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207 **Equine anti-igG conjugate.** For evaluation of transfection of equine F(ab')₂ against RABV by
208 direct immunofluorescence, Anti-Horse IgG (whole molecule) -FITC antibody (Sigma-
209 Aldrich) was used.

210

211 **Virus.** Strain (IP3629/11-AgV2 dog isolated from Brazil), grown on mice central nervous
212 system (CNS) with a titre of $10^{3.8}$ DL₅₀/ μ L, kindly provided by the Pasteur Institute, Brazil,
213 was used for the infection in mice.

214

215 **Antibody transfection test.** FITC-antibody control protein (Gelantis) complexed with
216 Bioporter® Protein Delivery Reagent (Genlantis) per manufacturer's instructions were injected
217 by the intracerebral route in two mice and the CNS of each mouse was collected at 4 and 6
218 hours post-infections; 10 μ m sections were obtained in a LEICA CM 1860 UV microtoime,
219 fixed in glas sliced with -20°C acetone/2 hours and observed for fluorescence with a
220 OLYMPUS ® BX53 epifluorescence microscope.

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222 **Direct fluorescent antibody test (DFAT) and PCR.** All mice were observed for period 30 days
223 after RABV inoculation for signs of rabies such as anorexia, piloerection hyperesthesia,
224 aggressiveness, paralysis and death, being the surviving mice euthanized at the end of the
225 observation period. The central nervous system (CNS) of each mouse was tested with a direct
226 fluorescent antibody test (DFAT) [24], using an anti-RABV nucleocapsid IgG conjugates with
227 Fluorescein isothiocyanate (Pasteur Institute, Brazil), and, if negative, to a PCR targeting
228 RABV N-P genes [25].

229

230 **Statistical analysis.** GraphPad Prism was used for statistical analyses of *in vivo* data. Fisher's
231 test with $\alpha = 0.05$ was used for the statistical analysis with Fisher Exact Test Calculator online.

232

233 **Acknowledgments.** The authors are thankful for the financial support provided by CNPq
234 (Brazilian National Board for Scientific and Technological Development) grant number
235 307291/2017-0 and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior,

236 Brazil) Finance Code 001, which had no role in the study design, collection, analysis and
237 interpretation of data, writing of the report and in the decision to submit the article for
238 publication.

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357 Fig. 1 - Ten- μ m cryosections of mice brain after transfection of antibodies with FITC-antibody control protein®
358 (Genlantis) as a transfection tracer in the central nervous system of mice using Bioporter® (Genlantis) as a
359 transfection agent showing fluorescent dots (arrows) on the cytoplasm at 4 (A) and 6 (B) hours post-injection.
360 200x increase.

361

362 Fig. 2 - Survival plot for mice inoculated intracerebrally with $10^{3.8}$ DL_{50%} RABV DOG-IP3629/11 and treated 48
363 h post-inoculation with Anti-RABV F(ab')₂ plus Bioporter ® (solid line) or Anti-RABV F(ab')₂ plus Hepes 20
364 mM pH 7.4 (dashed line).



