1 **Glutathione is a highly efficient thermostabilizer of poliovirus Sabin strains**

2 Running title: Thermostabilization of poliovirus by glutathione

3 *Rana Abdelnabi, Leen Delang, Johan Neyts*

4 KU Leuven – University of Leuven, Department of Microbiology and Immunology, Rega
5 Institute for Medical Research, Laboratory of Virology and Chemotherapy, B-3000
6 Leuven, Belgium.

7

8 *Corresponding author: Johan Neyts, Herestraat 49, 3000 Leuven, Belgium. Tel

9 +3216321105, email: Johan.Neyts@kuleuven.be.

Abstract

Glutathione (GSH) is the most abundant thiol peptide in animal cells and has a critical role in antioxidation. GSH was reported to be essential for stabilization of some enteroviruses, including poliovirus (PV), during viral morphogenesis. Here, we explored the potential use of GSH as a thermostabilizer of oral poliomyelitis vaccine (OPV) formulations. GSH significantly protected the three types of PV from heat-inactivation in a concentration-dependent manner. At a GSH concentration of 20 mM, nearly complete protection was observed against heating temperatures up to 53°C for 2 min. GSH also markedly protected PV1 from heat-inactivation and this up to 6 hours at temperatures of 44°C and 46°C and 3 hours at 48°C. The fact that GSH is naturally present at high concentration in the human body makes it an efficient candidate stabilizer for OPV formulations.

Key words

Glutathione; poliomyelitis; cold chain; vaccine; Sabin

24 **Highlights**

- 25 • Glutathione (GSH) is an essential host factor for stabilization of poliovirus during viral
26 morphogenesis.
- 27 • Glutathione increased the thermostability of poliovirus Sabin strains in a
28 concentration-dependent manner.
- 29 • The thermostabilization by GSH was also observed after prolonged periods of
30 incubation at temperatures up to 50°C.

31

Glutathione (GSH) is the most abundant thiol peptide in animal cells, it has a critical role in antioxidation, detoxification of xenobiotics and maintenance of immune system functions [1–3]. GSH is a tripeptide composed of glutamine, cysteine and glycine, which is readily oxidized into the disulfide form (GSSG) in a non-enzymatic reaction [2]. The imbalance of the ratio of reduced and oxidized forms of glutathione (GSH/GSSG) is observed in several human diseases such as cancer, neurodegenerative diseases and some viral infection such as those caused by HIV-1 [2,4–6]. Our laboratory demonstrated earlier that GSH is an essential host factor for stabilization of Coxsackievirus B3 (CVB3), an enterovirus, during viral morphogenesis. Selective and rapid depletion of intracellular GSH level results in a potent anti-enterovirus effect [7]. Enteroviruses C, including poliovirus, also require GSH during their morphogenesis [8].

The oral polio vaccine (OPV) has been widely and successfully used in the poliomyelitis eradication programs. Because of its sensitivity to temperature, this live-attenuated vaccine needs a strict cold-chain for storage and transport [9]. Several strategies have been explored to increase the thermostability of OPV such as the use of $MgCl_2$ [10], sucrose [11], heavy water (D_2O) [12], capsid binders [13] and fatty acids [14]. Currently, $MgCl_2$ is the most widely used stabilizer for OPV formulations [15]. However, some OPV formulations are also stabilized with sucrose (35-50%) [15]. The half-life of stabilized OPV is 48h at 37°C, 24h at 41°C, 14h at 45°C and 3h at 50°C [9,15]. Here, we wanted to explore the effect of GSH on the thermostability of different types of PV vaccine strains and thus the possible use of GSH as a new stabilizer for oral poliovirus vaccine (OPV) formulation. This may allow manipulation of OPV out of the cold-chain for longer periods of time which will reduce the cost for vaccine transport, especially in non-developed countries.

First, to explore whether GSH has the potential to serve as a thermostabilizer for the oral polio vaccine, the dose-response effect of GSH on the thermostability of the three types of

poliovirus Sabin strains (PVs) was studied. Briefly, PV1, PV2 and PV3 (TCID₅₀/ml= 2x10⁶, 2x10⁵ and 1.4x10⁵, respectively) were incubated with different GSH concentrations (2-50 mM, prepared in PBS) for 30 min at 48°C. The three types of PV were markedly protected from heat-inactivation in a concentration-dependent manner (Fig.1). At a concentration of 20 mM or higher, GSH resulted in near complete protection of the infectivity of the three strains. For this reason, 20 mM of GSH was selected for subsequent studies (Fig.1). The pH of GSH solutions of 20 mM, 10 mM, 5 mM and 2 mM is respectively 3.6; 4.0; 6.2 and 6.6. As shown in (Fig.1), the stabilizing effect of 5 mM GSH solution on PV1 and PV2 is comparable to that of 10 and 20 mM GSH solutions. It thus appears that the thermostabilizing effect of GSH is pH independent and that this stabilizing effect is mainly due to the direct interaction of the molecule with the viral capsid [7]. The level of GSH in erythrocytes of infants is about 9 nmol/mg hemoglobin (Hb) [16]. This corresponds to ~130 mg total GSH in the blood (~280 ml) of a 4 kg weighing infant. Infants have also an intake of GSH via breastmilk. The average GSH concentration in breastmilk is ~192 µmol/L (=59 mg/L) [17]. Therefore, the concentration of GSH used in this study (20 mM or 6.2 mg/ml would correspond to 3.1 mg per OPV dose which is equivalent to the GSH content in 53 ml breastmilk. The effect of 20 mM GSH on the thermostability profile of PV was then determined by incubating PV1, PV2 and PV3 Sabin strains at different temperatures [range: 42 to 56°C] for 2 min followed by rapid cooling at 4°C. GSH markedly protected the 3 types of poliovirus vaccine strains from heat-inactivation at all the tested temperatures (Fig.2A-C). At the highest studied temperature (56°C), GSH resulted in 100% protection of PV2 infectivity against heat-inactivation compared to the untreated control (Fig.2B). Also at 56°C, GSH still resulted in a markedly protective effect where by 79% of PV1 (Fig.2A) and 60% of PV3 (Fig.2C) initial titers were recovered.

81 Next, the time-dependency of the stabilizing effect of GSH was determined; to that end
82 PV1 was incubated at either 44°C, 46°C, 48°C and 50°C over a time span of 6 hours in the
83 presence or absence of 20 mM GSH. Samples were collected every hour to quantify the
84 remaining infectious viral load. GSH markedly protected PV1 from heat-inactivation for
85 prolonged periods of time. Indeed, respectively 92 and 83% of the initial virus titers were
86 recovered after 6 hours of incubation at 44°C (Fig.3A) and 46°C (Fig.3B). Whereas heating
87 of the untreated PV1 for two hours at 48°C or one hour at 50°C resulted in a complete loss
88 of the infectivity of the virus; 20 mM GSH resulted in 84 and 45% recovery of the initial
89 PV1 titers following 3 hours of incubation at 48°C and 50°C, respectively (Fig.3C/D).

90 In 1988, the Global Polio Eradication Initiative (GPEI) has been launched by WHO, these
91 efforts reduced the number of PV cases by >99% [18]. OPV has been widely used for polio
92 eradication because of its lower cost, higher ability to induce intestinal immunity than the
93 inactivated polio vaccine (IPV) and ease of administration. However, the use of OPV is
94 associated with the risk of vaccine associated paralytic poliomyelitis (VAPP) due to
95 development of mutants with the neurovirulence and transmission characteristics of the
96 wild-type virus i.e. vaccine-derived polioviruses (VDPV) [19]. Therefore, the development
97 of genetically-stable modified Sabin strains is currently under study to increase the safety
98 of live attenuated polio vaccine. Recently, new PV2 Sabin strains have been developed and
99 proven to be genetically-stable and hyper-attenuated [de Boer SM, Macadam A., Andino
100 R., Burns C. and Bakker W. (Europic2016, Switzerland, 4 to 8 September 2016)]. Because
101 of its safety and efficiency as a PV-thermostabilizer, GSH may serve as candidate stabilizer
102 for such new OPV formulations.

103 **Funding information**

104 This work was funded by a grant for the BELVIR project from BELSPO (IUAP) and the EU
105 FP7 Industry-Academia Partnerships and Pathways Project AIROPICO. L.D. was
106 supported by a post-doctoral fellowship from the FWO (Fund for Scientific Research of
107 Flanders, Belgium).

108

109 **Conflict of interest:** Non to declare

110

References

- [1] Fraternale A, Paoletti MF, Casabianca A, Nencioni L, Garaci E, Palamara AT, et al. GSH and analogs in antiviral therapy. *Mol Aspects Med* 2009;30:99–110. doi:10.1016/j.mam.2008.09.001.
- [2] Lagman M, Ly J, Saing T, Singh MK, Tudela EV, Morris D, et al. Investigating the causes for decreased levels of glutathione in individuals with type II diabetes. *PLoS One* 2015;10. doi:10.1371/journal.pone.0118436.
- [3] Marí M, Morales A, Colell A, García-Ruiz C, Fernández-Checa JC. Mitochondrial glutathione, a key survival antioxidant. *Antioxid Redox Signal* 2009;11:2685–700. doi:10.1089/ARS.2009.2695.
- [4] Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, et al. Role of glutathione in cancer progression and chemoresistance. *Oxid Med Cell Longev* 2013. doi:10.1155/2013/972913.
- [5] Morris D, Guerra C, Donohue C, Oh H, Khurasany M, Venketaraman V. Unveiling the mechanisms for decreased glutathione in individuals with HIV infection. *Clin Dev Immunol* 2012;2012. doi:10.1155/2012/734125.
- [6] Johnson WM, Wilson-Delfosse AL, Mieyal JJ. Dysregulation of glutathione homeostasis in neurodegenerative diseases. *Nutrients* 2012;4:1399–440. doi:10.3390/nu4101399.
- [7] Thibaut HJ, van der Linden L, Jiang P, Thys B, Canela MD, Aguado L, et al. Binding of Glutathione to Enterovirus Capsids Is Essential for Virion Morphogenesis. *PLoS Pathog* 2014;10. doi:10.1371/journal.ppat.1004039.
- [8] Ma HC, Liu Y, Wang C, Strauss M, Rehage N, Chen YH, et al. An Interaction between Glutathione and the Capsid Is Required for the Morphogenesis of C-Cluster Enteroviruses. *PLoS Pathog* 2014;10. doi:10.1371/journal.ppat.1004052.
- [9] Shrivastava A, Gupta N, Upadhyay P, Puliyl J. Caution needed in using oral polio vaccine beyond the cold chain: Vaccine vial monitors may be unreliable at high temperatures. *Indian J Med Res* 2012;135:520–2.
- [10] Wallis C and Melnick JL. Stabilization of poliovirus by cations. *Tex Rep Biol Med* 1961;19:683–700.
- [11] Mirchamsy H, Shafyi A, Mahinpour M, Nazari P. Stabilizing effect of magnesium chloride and sucrose on Sabin live polio vaccine. *Dev Biol Stand* 1978;41:255–7.
- [12] Wu R, Georgescu MM, Delpeyroux F, Guillot S, Balanant J, Simpson K, et al.

- Thermostabilization of live virus vaccines by heavy water (D2O). *Vaccine* 1995;13:1058–63. doi:10.1016/0264-410X(95)00068-C.
- [13] Andries K, Rombaut B, Dewindt B, Boeyé a. Discrepancy between infectivity and antigenicity stabilization of oral poliovirus vaccine by a capsid-binding compound. *J Virol* 1994;68:3397–400.
- [14] Dorval BL, Chow M, Klibanov AM. Stabilization of poliovirus against heat inactivation. *Biochem Biophys Res Commun* 1989;159:1177–83. doi:10.1016/0006-291X(89)92234-1.
- [15] Milstien JB, Galazka AM, Kartoglu U, Zaffran M. Temperature sensitivity of vaccines. *WHO Geneva* 2006:1–58. doi:WHO/IVB/06.10.
- [16] Lee Y, Chou Y. Antioxidant Profiles in Full Term and Preterm Neonates. *Chang Gung Med J* 2005:846–51.
- [17] Ankrah N, Appiah-opong R, Dzokoto C. Human Breastmilk Storage and the Glutathione Content. *J Trop Pediatr* 2000:111–3.
- [18] Moturi EK, Porter KA, Wassilak SGF, Tangermann RH, Diop OM, Burns CC, et al. Progress toward polio eradication - worldwide, 2013-2014. *MMWR Morb Mortal Wkly Rep* 2014;63:468–72. doi:10.1093/infdis/jit808.
- [19] Orenstein WA, Committee on Infectious Diseases TCOI, Sutter R, Kew O, Cochi S, Aylward B, et al. Eradicating polio: how the world's pediatricians can help stop this crippling illness forever. *Pediatrics* 2015;135:196–202. doi:10.1542/peds.2014-3163.

Figures

Fig.1. Concentration-dependent heat-stabilization of PVs by glutathione.

Polioviruses types 1, 2 and 3 were incubated with increasing concentrations of glutathione (GSH) at 48°C for 30 min, after which samples were stored on ice until the remaining infectious viral load was quantified by endpoint titration and converted into % of the pre-incubation TCID₅₀/ml. The data presented are the average $\pm$ SD of three independent experiments. Significant differences between the control and GSH-treated virus were assessed using the unpaired Student's t-test (*P<0.05, **P<0.01, ***P=0.007). Statistical analysis was performed using the Prism software (GraphPad).

179

180 **Fig.2. Effect of glutathione on the thermostability profile of polioviruses**

181 Polioviruses types 1 (A), 2 (B) and 3(C) were heat-inactivated for 2 min at the specified
 182 temperatures in presence or absence of 20 mM GSH after which samples were stored on
 183 ice the infectious virus content in each sample was quantified by endpoint titration. The
 184 dotted lines represent 50% of the TCID₅₀/ml prior to incubation. Data are the average $\pm$
 185 SD of three independent experiments. Significant differences from the untreated control
 186 were determined by the Prism software (GraphPad) using the unpaired Student's t-test
 187 (*P<0.05, **P<0.01, ***P<0.005).

Fig.3. Prolonged heat-stabilization of poliovirus type 1 by GSH

Poliovirus type 1 (PV1) was incubated at either A) 44°C, B) 46°C, C) 48°C or D) 50°C for 6 hours in presence or absence of 20 mM GSH. Samples were collected every hour and after cooling on ice, the remaining infectious viral load was determined by endpoint titration. The dotted lines represent 50% of the TCID₅₀/ml prior to incubation. The data are the average $\pm$ SD of three independent experiments. Significant differences from the untreated control were assessed using the unpaired Student's t-test (*P<0.05, **P<0.01, ***P<0.005, ****P<0.001). Statistical analysis was performed using the Prism software (GraphPad).