

Herpes Simplex Virus Type I Infection of Mice Testis and Effect on Fertility

Victor A. Naumenko, Yuriy A. Tyulenev, and Alla A. Kushch

Abstract—The objective of current issue was to develop a model of testicular herpes simplex virus (HSV) type I infection for assessment of viral effect on fertility. 56 male mice were inoculated intraperitoneally with different concentrations of HSV on 8 day post partum. It was revealed that the optimal dose was 100 plaque forming units per mice as it provided testicular infection in 100% of survivors. HSV proteins were detected both in somatic and germ cells (spermatogonia, spermatocytes, spermatides). Although DNA load in testis was descending from 3 to 28 days post infection only 12.5% of infected males had offspring after mating with uninfected females comparing to 87.5% in control ($p=0.012$). These results are the first direct evidence for HSV impact in male sterility. Prepuberal mice appeared to be a suitable model for investigation of pathogenesis of virus-associated fertility disorders.

Keywords—Herpes simplex virus type I, male fertility, prepuberal mice, spermatogenesis.

I. INTRODUCTION

VIRAL infection of the testis is a major health problem as it can cause sterility. Several investigators have declared correlation between herpes simplex virus (HSV) infection of human sperm and male fertility disorders. Up to 49.5% [1] of men consulting for infertility demonstrated HSV DNA in sperm samples.

Borai *et al.* [2] have found HSV DNA in 24% of sperm samples from patients of infertility clinics but none from healthy men. Using in situ hybridization Kotronias *et al.* [3] have detected HSV in the sperm of infertile and fertile men in 46% and 17% respectively, and it was found the decrease in sperm concentration and motility in infected samples [3], [4]. Previously we have shown that prevalence of HSV in sperm is higher in men with fertility disorders and correlates with reduced sperm parameters [5]. Frequency of HSV detection in sperm samples of partners of women with repeated miscarriages was found to be higher than in the control group that could be interpreted as indirect evidence for a vertical HSV transmission [6].

On the other hand, relationship between HSV and infertility is not commonly accepted [7], [8] and, in fact, data obtained provide only indirect evidence for the HSV impact on male sterility as another factors cannot be excluded so far. The attempts to solve the problem are hampered by the absence of appropriate experimental model because mature spermatozoa

are resistant to HSV-infection and immature germ cells are protected by hematotestis barrier (HTB).

It has been well defined that HTB is absent in newborn mice and its formation is associated with appearance of meiotic cells on 15-17 days post partum (dpp) [9]. The main challenge of the work was to obtain testicular HSV infection before HTB assembly and to investigate prospectively the fertility of infected males.

II. MATERIALS AND METHODS

A. Animals

56 male DBA mice were inoculated intraperitoneally with 100 μ l of HSV type I at 8 dpp and 16 mock-infected males were used as a control. From these animals 8 infected and 8 intact males were mated with 32 uninfected females.

B. Virus

HSV type I strain F was used for inoculation in 3 doses and the animals were grouped according to this: 10^4 plaque forming units (PFU/ml; Group I, $n=7$), 10^3 PFU/ml (Group II, $n=29$) and 10^2 PFU/ml (Group III, $n=20$). The optimal dose was determined as viral concentration that gave the highest percent of testicular infection in survivors.

C. HSV Type I Detection in Inoculated Males

On 3, 8, 18, 28 days post infection (dpi) mice were sacrificed (2-3 infected and mock-infected males at each time point). Testis, brain and kidneys (pooled right and left) from these animals as well as from mice that died from HSV-infection were removed, weighed and homogenized in 1000 μ l of medium. HSV DNA extraction was performed from 200 μ l of samples using QIAamp DNA mini kit (QIAGEN) according to the manufacturer's protocol and Amplisense HSVI, II-FL kit (Interlabservice) was used for real-time PCR (rt-PCR). Quantification of HSV DNA was realized by including of two standard positive samples in the run and by normalization of DNA copies to the weight of the probe. HSV proteins were detected in slides from formaldehyde-fixed paraffin-embedded testis using anti-HSV-I,II rabbit polyclonal antibodies (Abcam).

D. Mating and Assessment of Fertility

On 42 dpp 16 males (8 infected and 8 mock-infected) were placed with 32 females (2 females per male) for 2 days and after it the males were sacrificed. Testis were removed, weighed and prepared for rt-PCR and immunostaining as described above. The percentage of males that had offspring

V. N, Yu. T and A. K are with the D. I. Institute of Virology of Ministry of Health of Russian Federation, 123098, 16 Gamaleya str., Moscow, Russia (phone: 499-190-30-49; e-mail: virol8@mail.ru).

as well as the number of newborns in infected and control groups were used as criteria for fertility assessment.

E. Statistics and Data Analysis

The analysis was performed in StatXact 8 using Student's t-test and χ^2 test. $P < 0.05$ was considered statistically significant.

III. RESULTS

A. Clinical Outcome

All of mock-infected mice as well as mice of infected Group III remained alive and demonstrated no clinical signs of HSV-infection. In contrast all males of Group I died on 2-4 dpi. In Group II all males have clinical signs of HSV-infection such as bristling of fur and 12 out of 29 (41%) died on 2-11 dpi with the severe signs of viral meningitis (Group IIa) whereas 17 males (59%) survived (Group IIb).

B. HSV Type I Detection in Inoculated Male Mice

Rt-PCR was used to define whether testicular HSV-infection was obtained or not after intraperitoneal inoculation and it considered to be approved if one or both testis contained viral DNA. In Group I and Group IIa HSV DNA was detected in 86% (6/7) and in 100% (12/12) of testis respectively. All brain and kidneys samples in these groups also contained viral DNA. Spacious foci of immunostained interstitial cells, spermatogonia and Sertoli cells were found in testis samples (data not shown).

In testis samples obtained from Group IIb and Group III on 3-28 dpi viral DNA was found in 100% (9/9) and 10% (2/20) of males respectively ($p < 0.001$). We did not find HSV DNA in any brain sample from survivors (Group IIb and III) and kidneys samples in these animals appeared to be positive in 44% (4/9) and 5% (1/20) respectively ($p = 0.04$).

As Group II demonstrated testicular HSV-infection in 100% of males and 59% of mice remained alive, we considered 100 μ l intraperitoneal injection of 10^3 PFU/ml to be the optimal infection procedure for the goal of the study and further we used only survivors from this group (IIb).

C. Dynamics of Testicular HSV-infection

Fig. 1 demonstrates dynamics of HSV infection in Group IIb as it was found by PCR. One can see that viral load in testis declined from 3-8 to 28 dpi. It should be noted that initial viral load in testicular tissue (10^3 copies/mg) is much more lower than that of the Group I (1.9×10^5 copies/mg) and Group IIa (1.4×10^4 copies/mg). Unexpectedly it was found an asymmetry in viral load of left and right testis: it was higher in the right one in Group IIb. Moreover analysis of PCR results in all three groups revealed that among 29 males with testicular HSV infection 10 had viral DNA only in the left testis (34.5%), 1 – only in the right one (3.4%, $p = 0.007$), whereas 18 (62.1%) – in both testis. Immunostaining was used to confirm that HSV did infect testicular cells. In fact we found viral proteins in interstitial cells and in seminiferous tubules.

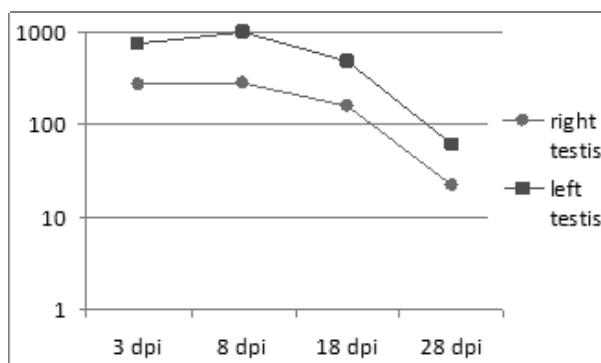


Fig. 1 Dynamics of HSV DNA in infected mice testis. Horizontal axis – days post infection (dpi); vertical axis – viral load (copies/mg)

In 2 cases several tubules were infected entirely, but more often only few infected cells (spermatogonia, spermatocytes or spermatides) were found (Fig.2). Neither HSV DNA nor viral proteins were found in mock-infected males.

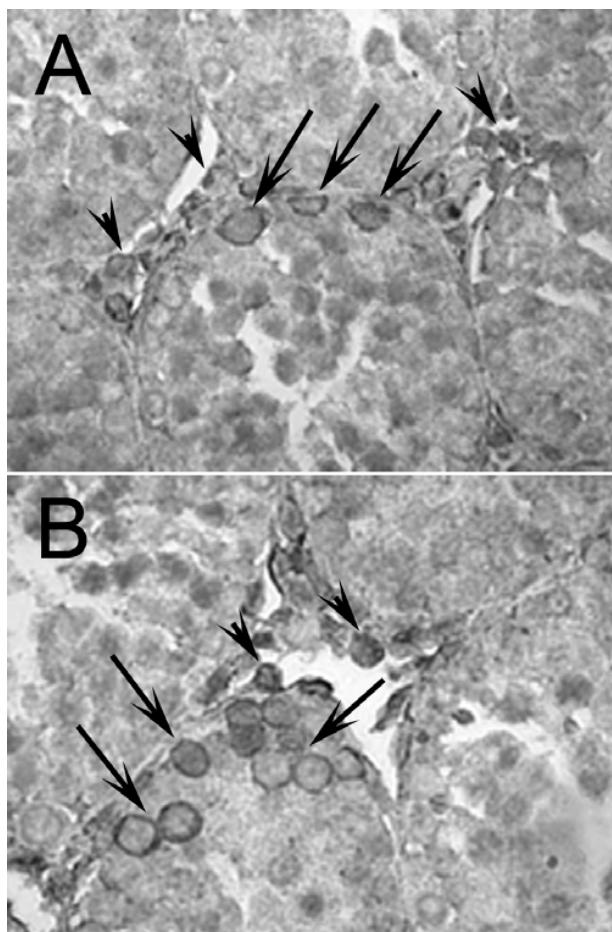


Fig. 2 Detection of HSV proteins in interstitial (arrowheads) and germ (arrows) cells on 8 (A) and 18 (B) dpi. Magnification x400

D. Assessment of Fertility

The parameters that were used for assessment of male

fertility are summarized in Table I. 9 from 16 females that were placed with mock-infected males had a total number of 55 newborns (6 newborns per female on average) and percentage of males that had offspring in this group was found to be 87.5% (7/8). By contrast only one female had 4 newborns in the Group of females that mated with HSV-infected males that resulted in 12.5% (1/8, $p=0.012$) of fertile males.

TABLE I
COMPARATIVE ANALYSIS OF FERTILITY IN MOCK-INFECTED AND HSV-INFECTED MALE MICE

Parameters	Groups of animals	
	Uninfected females + mock-infected males	Uninfected females + HSV-infected males
Medium testis weight, g	0.094*	0.081*
Total number of newborns	55	4
Fertile males, %	87.5**	12.5**

* $p=0.01$ (Student's test); ** $p=0.012$ (χ -squared test)

The weight of the males in both groups did not differ (21.9 g vs 22 g, $p>0.05$) whereas the weight of the testis of infected mice were lower comparing with control (0.094 g vs 0.081 g, $p=0.01$). No virus was found in brains of infected mice, but in 75% probes (6/8) virus was detected in testis or in kidney, namely 25% of samples (2/8) demonstrated viral DNA in left testis and 50% (4/8) – in kidneys. It should be mentioned that viral load in testis (3×10^5 copies/mg) and in kidneys (2.8×10^4 copies/mg) was even higher than that of the samples from Group IIb at the beginning of infection. Interestingly, the only male that had offspring in HSV-infected group was found free of virus.

IV. DISCUSSION

Viruses can enter the testis either via the blood and lymphatic vessels that present in the interstitial cells or via the rete testis. Intratesticular injection is a common way of testicular infection modeling [10], [11], but this procedure can lead to autoimmune orchitis by itself. Still there is no definite answer if herpes viruses infect germ cells [10] or only interstitial cells [11] after intratesticular injection. It was demonstrated that hematogenous way of testicular infection in adult animals is hardly performed due to the HTB [12]. In the current issue we developed an original way to solve the problem – after intraperitoneal injection virus entered the testis via blood stream in prepuberal mice that lacked HTB. Another challenge for investigation of HSV-infection in newborns follows from the results of Burgos *et al* [13]. It was shown in mice that HSV type I infected mothers can transmit virus via blood stream during delivery and as a result HSV can be found in newborns in several tissues including testis.

Among three doses of infection we used 100 PFU per/mice

(Group II) gave the best results in achieving testicular infection in survivors: 100% of males demonstrated HSV DNA in testis and 59% remained alive. In contrast with previous results [13] we found HSV DNA in brains only in lethal outcomes whereas kidneys were found to be infected in 44% of Group II survivors. In all three groups frequency of HSV detection in left testis was significantly higher than that of the right testis (97% vs 66%, $p=0.008$). This asymmetry can be explained by the difference in blood perfusion in the right and left testis that results in higher efficiency of virus delivery to the left testis during viremia. HSV DNA was detected in the testis all over the experiment, but after 18 dpi it decreased significantly. In order to prove that DNA persisting in organ was due to the testicular infection immunostaining was performed. Both somatic and germ cells (spermatogonia, spermatocytes, spermatids) were found to contain viral proteins. The descent in viral load during 28 dpi is a common feature of primary non-lethal HSV-infection as in 4 weeks after infection the latency gets established [14]. In these terms the increase in viral load that was found in males after mating (36 dpi) can be regarded as reactivation from latency.

The most intriguing results were obtained in mating experiment. We observed a drastic decrease in fertility rate in HSV-infected males: only 1 out of 8 males (12.5%) was found to be fertile comparing with 87.5% in control. To our knowledge this is the first reported direct evidence of HSV impact in male sterility. It should be noted that for 6 out of 7 males that had not offspring virus was found in urogenital organs: testis (2 cases) and kidneys (4 cases). This paper did not target pathogenetic mechanisms of HSV-induced fertility disorders, but one can suppose several pathways.

Early in postnatal life the first phase of spermatogenesis (up to 35 dpp) is accompanied by an initial wave of germ cells apoptosis [15]. This period is extremely important for the state of future fertility. Hyperstimulation of both pro- and antiapoptotic pathways can lead to severe disturbance of spermatogenesis [15] and it is well known that HSV is able to induce as well as to inhibit apoptosis in infected cells [16]. Moreover viral infection can induce overexpression of several cytokines among which at least 2 molecules – IFN- γ and TNF- α – have hametotoxic effects [17], [18]. In polyoma-induced orchitis degeneration of the seminiferous epithelium was associated with decreased testosterone and increased luteinizing hormone serum concentrations as well as with anti-sperm antibodies detection [19]. However, we must consider the possibility that fertility disorders in infected males are transient and that the mice can return to normal fertility in 2 or 3 month. Further investigation of these issues is very important for understanding of HSV influence on male fertility.

REFERENCES

- [1] N. Kapranos, E. Petrakou, C. Anastasiadou and D. Kotronias, "Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic," *Fertil Steril.*, vol. 79, suppl. 79, pp. 1566-1570, Jun 2003.

- [2] N. el Borai, M. Inoue, C. Lefevre, E. N. Naumova, B. Sato and M. Yamamura, "Detection of herpes simplex DNA in semen and menstrual blood of individuals attending an infertility clinic," *J Obstet Gynaecol Res.*, vol. 23, pp. 17-24, Feb 1997.
- [3] D. Kotronias and N. Kapranos, "Detection of herpes simplex virus DNA in human spermatozoa by in situ hybridization technique," *In Vivo.*, vol. 12, pp 391-394, Jul-Aug 1998.
- [4] G. Bezold, J.A. Politch, N.B. Kiviat, J. M. Kuypers, H. Wolff and D. J. Anderson, "Prevalence of sexually transmissible pathogens in semen from asymptomatic male infertility patients with and without leukocytospermia," *Fertil Steril.*, vol. 87, pp. 1087-1097, May 2007.
- [5] R. R. Klimova, E.V. Chichev, V. A. Naumenko, Z. S. Gadzhieva, A. S. Tsibisov, et al., "Herpes simplex virus and cytomegalovirus in male ejaculate: herpes simplex virus is more frequently encountered in idiopathic infertility and correlates with the reduction in sperm parameters," *Vopr virusol.*, vol. 57, pp. 27-31, Jan 2010.
- [6] E. N. Bocharova, L. E. Zavalishina, E. E. Bragina, R. R. Klimova, Y. K. Gusak, et al., "Detection of herpes simplex virus genomic DNA in spermatozoa of patients with fertility disorders by in situ hybridization," *Dokl Biol Sci.*, vol. 412, pp. 82-86, Jan 2007.
- [7] G. Bezold, A. Schuster-Grusser, M. Lange, H. Gall, H. Wolff, et al., "Prevalence of human herpesvirus types 1-8 in the semen of infertility patients and correlation with semen parameters," *Fertil. Steril.*, vol. 76, pp. 416-418, Aug 2001.
- [8] E. Neofytou, G. Sourvinos, M. Asmarianaki, D. A. Spandidos, A. Makrigiannakis, "Prevalence of human herpes virus types 1-7 in the semen of men attending an infertility clinic and correlation with semen parameters," *Fertil. Steril.*, vol. 91, pp. 2487-2494, Jun 2009.
- [9] A. R. Bellve, J. C. Cavicchia, C. F. Millette, D. A. O'Brien, Y. M. Bhatnagar, et al., "Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization," *J. Cell Biol.*, vol. 74, pp. 68-85, Jul 1977.
- [10] S. V. Gribencha, E. E. Bragina, R. A. Abdumalikov, E. N. Bocharova, L. F. Kurilo, "Detection of type 2 herpes simplex virus in cells of spermatogenic epithelium in infected testis of guinea pigs", *Bull. Exp. Biol. Med.*, vol. 144, pp. 73-76, Jul 2007.
- [11] L. Tebourbi, A. M. Courtot, R. Duchateau, A. Loeuillet, J. Testart, et al., "Experimental inoculation of male mice with murine cytomegalovirus and effect on offspring," *Hum. Reprod.*, vol. 16, pp. 2041-2049, Oct 2001.
- [12] J. S. Burgos, C. Ramirez, I. Sastre, J. M. Alfaro, and F. Valdivieso, "Herpes simplex virus type 1 infection via the bloodstream with apolipoprotein E dependence in the gonads is influenced by gender," *J. Virol.*, vol. 79, pp. 1605-1612, Feb 2005.
- [13] J. S. Burgos, C. Ramirez, F. Guzman-Sanchez, J. M. Alfaro, I. Sastre, et al., "Hematogenous vertical transmission of herpes simplex virus type 1 in mice," *J. Virol.*, vol. 80, no. 6, pp. 2823-2831, Mar 2006.
- [14] C. S. Miller, R. J. Danaher, R. J. Jacob, "Molecular aspects of herpes simplex virus I latency, reactivation, and recurrence," *Crit Rev Oral Biol Med.*, vol. 9, no. 4, pp. 541-562, Jan 1998.
- [15] C. Shaha, R. Tripathi, D. P. Mishra, "Male germ cell apoptosis: regulation and biology," *Philos Trans R Soc Lond B Biol Sci.*, vol. 365, pp. 1501-1515, May 2010.
- [16] M. Aubert, J. O'Toole, J. A. Blaho, "Induction and prevention of apoptosis in human HEp-2 cells by herpes simplex virus type 1," *J Virol.*, vol. 73, no. 12, pp. 10359-10370, Dec 1999.
- [17] A. P. Satie, S. Mazaud-Guittot, I. Seif, D. Mahé, Z. He, et al., "Excess type I interferon signaling in the mouse seminiferous tubules leads to germ cell loss and sterility," *J Biol Chem.*, vol. 286, no. 26, pp. 23280-23295, Jul 2011.
- [18] O. Sarkar, J. Bahrainwala, S. Chandrasekaran, S. Kothari, P. P. Mathur, et al., "Impact of inflammation on male fertility," *Front Biosci (Elite Ed)*, vol. 1, no. 3, pp. 89-95, Jan 2011.
- [19] S. Fountain, M. K. Holland, L. A. Hinds, P. A. Janssens, P. J. Kerr, "Interstitial orchitis with impaired steroidogenesis and spermatogenesis in the testes of rabbits infected with an attenuated strain of myxoma virus," *J Reprod Fertil.*, vol. 110, no. 1, pp. 161-169, May 1997.