Reverse Transcriptase Affects Gametogenesis and Preimplantation Development in Mouse

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Abstract. *Background/Aim: The expression of reverse transcriptase (RT) in ovaries, testes, gametes and embryos highlights its critical role in cell growth and differentiation. We sought to investigate the effects of the potent RT inhibitor lamivudine in gametogenesis and mouse embryo preimplantation development. Materials and Methods: Male and female FVB/N mice were treated with the reverse transcriptase inhibitor Lamivudine for seven consecutive weeks. Following treatment, mouse sperm parameters, testicular and ovarian morphology as well as post-IVF embryo development were evaluated. Results: Lamivudine impaired the sperm parameters and the testicular structure in male mice, the number of primordial germ cells and primary oocytes in ovaries of female mice, and the embryos' morphology and development up to the blastocyst stage during in vitro culture. Conclusion: The administration of lamivudine affected the processes of spermatogenesis and oogenesis as well as the in vitro preimplantation development of mouse embryos.*

The enzyme reverse transcriptase (RT) generates complementary DNA (cDNA) from an RNA template through a process called reverse transcription and has been primarily associated with the retroviral life cycle. In eukaryotic cells, RT activity is essential for the telomerase function and the mobilization of retrotransposable elements. Both telomerase and retrotransposons are highly expressed in transformed and undifferentiated cells, such as cancer and germ cells (1-6).

Telomerase is a ribonucleoprotein complex composed by a catalytic subunit of telomerase reverse transcriptase (TERT), telomerase RNA components (TERC) and binding proteins (7). It is localized at the end of eukaryotic chromosome and synthesizes telomeric repeats by reverse transcription. Telomerase activity has been observed in human fetal, newborn and adult testes and ovaries, but not in mature spermatozoa and oocytes (8). Specifically, high telomerase expression levels have been found in spermatogonia and in primary spermatocytes of adult testes, while decreased expression levels have been detected in secondary spermatocytes; however, no expression has been observed in spermatids and in epididymal or ejaculated spermatozoa (9). Regarding oocytes, telomerase is expressed at the early antral and preovulatory follicles, while it is significantly decreased during ovulation and oocyte maturation (7, 9).

Retrotransposons, a major type of genetic mobile elements that occupy 43% of the human and 37% of the mouse genome (10), encode RT and other proteins necessary for their mobilization. They are transcriptionally expressed in male and female gametes (11, 12), while heritable retrotranspositions have been documented in the germline and in embryos during the early stages of development (13). Finally, an RT activity, possibly of retrotransposon origin,

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has been revealed in mouse epididymal spermatozoa, which mediates the reverse transcription of exogenous RNA molecules to cDNA (14).

The RT has been a target of many antiretroviral drugs used in the treatment of HIV and hepatitis patients. Several studies have reported the negative effects of antiretroviral drugs on sperm parameters of HIV-infected patients, such as low ejaculate volumes, decreased sperm motility rates and high abnormal sperm morphology rates (15-17).

Taking into account the RT activity of the telomerase complex, the retrotransposition events during gametogenesis and early embryogenesis, the encoded transcriptase activity in mature spermatozoa as well as the impaired sperm parameters of HIV-patients undergoing antiretroviral therapy, we sought to explore the effects of the potent RT inhibitor lamivudine in mouse gametogenesis, oogenesis and embryo preimplantation development.

Materials and Methods

Animals. Inbred FVB/N mice were provided by the Breeding Laboratory Animal Institute (Ioannina University Medical School, Greece). Animals were kept under a controlled 12 h light–12 h dark life cycle with free access to standard diet and water. The General Directorate of Agricultural Economics and Veterinary Medicine of Epirus Region, Greece, approved the research protocols used. All research animals were treated in compliance with the European Union guidelines for the care and use of animals approved by our institution in accordance to principles of laboratory animal breeding and handling.

Study design. Thirty male and thirty female mice were randomly divided in two groups. The 1st group (Control group) received 0.2 ml of intraperitoneal saline and the 2nd group (lamivudine treated group) received 50 mg/kg of the nucleoside analogue RT inhibitor lamivudine for five consecutive days per week, for a total period of seven weeks (18). Lamivudine was diluted in dimethyl sulfoxide (DMSO) and injected in 0.2 ml saline. The same volume of DMSO, without lamivudine, was injected in 0.2 ml saline in control samples.

Histology examination. Testes and ovaries were fixed in neutral buffered formalin, embedded in paraffin, cut in 4µm thick sections and stained with hematoxylin–eosin. Histological examinations of tissues were conducted under light microscopy.

Sperm analysis. Spermatozoa collected from cauda epididymis were immediately transferred in culture medium (Sydney IVF Sperm Medium, COOK, Limerick, Ireland) under mineral oil (Sydney IVF Culture Oil, COOK, Limerick, Ireland) and were allowed to disperse in it by incubation for 20 min at 37°C in an atmosphere of 5% CO₂.

Shortly after the isolation, one drop of each sperm suspension was placed on a microscope slide and the motility was estimated microscopically at a magnification of 40×. After scoring two hundred spermatozoa per slide, the motility was classified as either of the following three classes: rapid progressive, slow progressive and non-progressive. Sperm count was calculated in well-mixed sperm suspensions using a Neubauer chamber. Morphological abnormalities, regarding head and tail defects, were assessed by

Papanicolaou staining. Two hundred spermatozoa per sample were counted and abnormal sperm percentages were calculated (19).

The sperm chromatin structure assay (SCSA) was used to determine fragmentation of sperm DNA, according to a protocol previously described (20). Spermatozoa were stained with Acridine Orange. The green (BP 530/30 filter) and red fluorescences (BP 650LP filter) were measured for 30,000 cells per sample after excitation with a 488 nm argon laser. Data analysis was performed by the FacsCalibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

In vitro fertilization experiments. In order to study its effect on mouse embryo preimplantation development, lamivudine was supplemented at a concentration of 200 µM (21) in fertilization medium and in cleavage medium from pronuclear to blastocyst stage. Lamivudine was diluted in DMSO and was added in culture media at a volume of 0.25%. The same volume of DMSO, without lamivudine, was added in media used in control samples.

In vitro fertilization process, including sperm preparation, ovarian stimulation and embryo preimplantation development, were carried out according to previously described protocol (22). In brief, sperm was collected from cauda epididymis and motile spermatozoa were selected using the Swim Up method. Sperm concentration was calculated using a Neubauer chamber. Sperm samples were left to be capacitated for 1.5 h (37°C, 5% CO₂) in the proper fertilization medium (Sydney IVF Fertilization Medium, COOK) under mineral oil. Mature oocytes were retrieved from the oviducts of female mice after ovarian stimulation with an intraperitoneal injection (IP) of 8 IU follicle stimulating hormone (rec-FSH, Gonal-F, Merk Serono, London, UK), followed by an IP injection of 5IU of human chorionic gonadotropin 48 hours later (hCG, Pregnyl, N.V. Organon, Oss, the Netherlands). The *in vitro* fertilization was achieved by incubating oocytes with sperm in the above-mentioned fertilization medium for 5h. Afterwards, the oocytes were washed in order to remove the excess spermatozoa. Fertilized embryos were cultured in cleavage and blastocyst medium (Sydney IVF Cleavage Medium & Sydney IVF Blastocyst Medium, COOK) until the blastocyst stage (37°C, 5% CO₂).

Results

The effect of Lamivudine on testis and ovarian histology. In order to study the effect of the reverse transcriptase (RT) inhibition on gametogenesis, the nucleoside analogue lamivudine was injected in male and female mice for five consecutive days per week, for a total period of seven weeks. After treatment, ovarian and testicular histological analysis was performed, while sperm parameters were evaluated.

Representative testicular and ovarian histological sections of the Control (A) and the lamivudine treated groups (B) are shown in Figures 1 and 2, respectively. The seminiferous tubule structure of lamivudine treated animals was found to be significantly impaired, while a noticeable reduction of Sertoli cells and germ cells of various differentiation stages was also observed.

The number of primordial germ cells was significantly reduced in the lamivudine treated female animals compared to the control group (6.50±1.38 vs. 22.33±3.72, $p<0.001$,

Figure 1. Testicular histological sections in the: A) control group and B) lamivudine treated group. Lamivudine was injected into male FVB/N mice at a concentration of 50 mg/kg for five consecutive days for a total period of seven weeks.

respectively). The number of primary oocytes was also lower in the lamivudine treated group compared to the control group (6.33 ± 2.16 vs. 9.83 ± 2.32 , $p=0.022$, respectively). However, no statistically significant difference was observed in the mean number of corpus luteum (lamivudine treated group: 6.83 ± 2.40 corpus luteum vs. control group: 5.00 ± 2.00 , $p>0.05$) and in the maximum ovarian diameter (lamivudine treated group: 2.83 ± 0.98 cm vs. Control group: 2.83 ± 0.41 cm, $p>0.05$) between the two groups. Finally, a slight increase in the number of atretic oocytes was observed in the lamivudine treated animals (4.50 ± 2.43 vs. 2.67 ± 1.21 , $p>0.05$, respectively).

The effect of lamivudine on sperm parameters. Sperm analysis was performed in cauda epididymis spermatozoa of the control and the lamivudine treated animals. Sperm concentration and motility were determined by microscopy (40 $\times$ magnification) shortly after sperm isolation. All sperm parameters presented noteworthy impairment in the lamivudine group compared to the control group. The epididymis sperm concentration in the control group was $31.67 \pm 3.67 \times 10^6$ spermatozoa/ml, while in the lamivudine treated group was only $22.78 \pm 2.22 \times 10^6$ spermatozoa/ml ($p>0.05$). In the control group, $49.05 \pm 3.82\%$ of the spermatozoa presented with rapid progressive motility,

Figure 2. Ovarian histological sections in the A) control group (40×) and B) lamivudine treated group (40×). Lamivudine was injected into female FVB/N mice at a concentration of 50 mg/kg for five consecutive days for a total period of seven weeks.

21.66±3.76% with slow progressive motility, 17.55±2.48% with slow circular movement, while the remaining 11.74±1.58% of the spermatozoa were immotile. In contrast, in the lamivudine treated group, only 7.22±1.73% of the spermatozoa presented with rapid progressive motility, 17.14±2.69% with slow progressive motility, 28.44±2.42% with slow circular movement, while the remaining 47.2±3.74% of the spermatozoa were immotile (Table I).

The morphology and nuclear chromatin integrity of the sperm cells were assessed by Papanicolaou staining (Figure 3) and chromatin structure assay, respectively. Increased rates of morphological abnormality were observed in the lamivudine treated group compared to the control group. Specifically, in the lamivudine group, only 22.93±1.73% of the spermatozoa had normal morphology, 45.93±2.75% had no tail, 24.13±1.05% had bent tail, 12.96±0.38% had coiled tail and 5.71±1.35% of them had head abnormalities. In contrast, in the control group, 75.14±1.63% of the spermatozoa presented with normal morphology, 6.69±1.27% with no tail, 12.31±2.99% with bent tail, 2.29±0.98% with

coiled tail and 3.58±0.56% with head abnormalities (Table I). The nuclear chromatin integrity was slightly decreased in the lamivudine treated animals compared to the control group (84.84±4.88 vs. 90.97±2.49, $p>0.05$).

The effect of lamivudine on mouse embryo preimplantation development. In order to address its impact on mouse embryo preimplantation development, lamivudine was added at a concentration of 200 μ M in the fertilization medium and in the cleavage medium during embryo development until the blastocyst stage.

The control group comprised 120 embryos, while the lamivudine treated group 110 embryos. The *in vitro* preimplantation development of mouse embryos was scored daily until the blastocyst stage. The developmental stages at which lamivudine exposed embryos were arrested are presented in Figure 3. A considerable number of these embryos had dramatically altered morphology and were arrested before the formation of blastocyst, indicating that the presence of the RT inhibitor negatively affected the

Table I. *Sperm parameters in the control and lamivudine treated groups.*

Sperm Parameters	Control group	Lamivudine treated group	p-Value
Concentration ($\times 10^6$ spermatozoa/ml)	31.67 $\pm$ 3.67	22.78 $\pm$ 2.22	ns
Nuclear chromatin integrity	90.97 $\pm$ 2.49	84.84 $\pm$ 4.88	ns
Motility (%)			
a. Rapid progressive	49.05 $\pm$ 3.82	7.22 $\pm$ 1.73	0.037
b. Slow progressive	21.66 $\pm$ 3.76	17.14 $\pm$ 2.69	ns
c. Non-progressive	17.55 $\pm$ 2.48	28.44 $\pm$ 2.42	ns
d. No motility	11.74 $\pm$ 1.58	47.2 $\pm$ 3.74	ns
Morphology (%)			
a. Normal	75.14 $\pm$ 1.63	22.93 $\pm$ 1.73	ns
b. Bent tail	12.31 $\pm$ 2.99	24.13 $\pm$ 1.05	0.033
c. No tail	6.69 $\pm$ 1.27	45.93 $\pm$ 2.75	0.05
d. Coiled tail	2.29 $\pm$ 0.98	12.96 $\pm$ 0.38	0.05
e. Head abnormalities	3.58 $\pm$ 0.56	5.71 $\pm$ 1.35	ns

All parameters are presented as mean value $\pm$ standard deviation. ns: Non-significant.

preimplantation development of embryos. Specifically, although 85% of the control embryos reached the blastocyst stage, only 55% of the lamivudine exposed embryos formed a blastocyst, while 34% of them were arrested at the stages of 2-10 cells.

Discussion

Reverse transcriptase (RT) mediates the reverse flow of genetic information from RNA to DNA. It is essential for the retrovirus lifecycle, the telomerase function and the intracellular retroelement replication in eukaryotic cells. The reverse transcriptase activity during gametogenesis has been associated with the telomerase activity and the retroelement expression, suggesting an essential role of RT function in the structural and functional maturation of ova and spermatozoa (8, 9, 11, 12, 14). To investigate the role of RT on gametogenesis, we administered the nucleoside analog reverse transcriptase inhibitor lamivudine in male and female mice for a total period of seven weeks. Testicular and ovarian structure defects, decreased sperm quality and impaired embryo preimplantation development were observed following treatment.

The administration of lamivudine affected the testicular and the ovarian tissue structure of the treated animals. Specifically, the seminiferous tubule cellular organization was significantly impaired in lamivudine treated animals possibly due to the remarkable reduction in the number of Sertoli cells. Extensive gaps along the whole tubule surface not only decreased the number of germ cells, which progressively develop and differentiate into mature gametes, but also reversed the movement of mature spermatids and spermatozoa from the

tubule lumen towards the basement membrane. The decrease in sperm concentration and the noteworthy alterations in the sperm motility and morphology reflect the impaired function of the seminiferous tubule. A considerable proportion of spermatozoa was immotile or was characterized by a slow circular movement. Regarding the morphological defects, most of them were observed in the midpiece or the neck of spermatozoa and more rarely in the head. Moreover, condensation of the sperm chromatin was slightly decreased in the lamivudine exposed group as compared to the control group. However, female mice of the lamivudine group presented with a significant decrease in the number of primary oocytes. Furthermore, an increase of atretic follicles was observed in the ovaries of the treated animals resembling those of women with PCOS. Finally, lamivudine impaired the *in vitro* preimplantation development of mouse embryos causing accelerated asymmetrical cell divisions, multiple cellular fragments and arrest at the stages of 2-10 cells.

Lamivudine as a nucleoside analog RT inhibitor probably exerted its side effects through the prevention of DNA polymerization leading to incomplete Sertoli cells proliferation and to inappropriate germ cell growth and differentiation. Additionally, the processes of gametogenesis and embryogenesis were possibly impaired through the inhibition of the retrotransposon transcription and the telomerase inactivation. Indeed, the functional inactivation of two RT encoded elements, LINE-1 and MuRV-1, and the exposure of mouse embryos in nevirapine, a non-nucleoside-analog reverse transcriptase inhibitor, have been shown to impair the early preimplantation development in mouse embryos (23), indicating the significant role of RT activity in embryogenesis. Additionally, it has been reported in *in vitro* studies that lamivudine and other commonly used antiretroviral drugs have significantly impaired telomerase activity and telomere length (24). Zivodune (AZT), a nucleoside analog RT inhibitor, has exhibited preferential incorporation into telomere sequences of immortalized cell lines, potentially telomerase mediated, leading to a marked progressive telomere shortening. An extended telomere length shortening may cause cell cycle arrest and cell replicative senescence (25-27). It has been reported that insufficient telomerase activity and short telomere length may adversely affect many cellular developmental events like chromosome segregation, viability, sperm population and fertility (28). Furthermore, absence of telomerase activity in abnormal oocytes has been associated with reproductive senescence and chromosomal abnormalities (9), while fertilized telomerase-deficient oocytes exhibit aberrant cleavage and embryo development. Finally, the lack of granulosa telomerase activity and the short telomeres in women have been correlated with ovarian insufficiency (7). Further studies are needed to examine the relationship between telomerase and retrotransposons' RT activity and to

Figure 3. A. Sperm cell and embryo morphology in the A) control group and B) lamivudine treated group. C. Developmental stage at which embryos of the control group and lamivudine treated group were arrested. Embryos were exposed to 200 μ M lamivudine from the fertilization process through to the blastocyst stage.

clarify if they act synergistically or not, in order to control the processes of gametogenesis and embryonic development.

Several studies have reported the adverse effects of antiretroviral drugs on sperm parameters of HIV-infected patients and specifically the decreased ejaculate volume, sperm motility and normal sperm morphology (15, 16, 29).

The study of the toxicological effects of nevirapine on Wistar rat testes has shown testicular lipid peroxidation (LPO) elevation that may be related to sperm morphology, motility and viability defects (30). Moreover, AF/ABDP cis, a rifampicin derivative, has induced ultrastructural alterations of pachytenes oocytes and mitochondrial damage in all ovarian

cells of *Xenopus laevis* (31). Finally, the administration of AZT in pregnant mice has resulted in embryo death during the first half of gestation, while the direct exposure of developing embryos to AZT has retarded embryo cell divisions and impaired developmental ability (32).

Our results agree with the findings of previous studies, verifying the negative effects of RT inhibitors on cell proliferation during gametogenesis. To conclude, the administration of lamivudine in male and female mice negatively affects the testicular/ovarian structure and function. Lamivudine causes seminiferous tubule structure disorganization, increased abnormal sperm morphology and DNA degradation, as found by the SCSA analysis of sperm. In the ovaries, the afore-mentioned inhibition leads to the formation of multiple cysts, increased preantral follicle numbers and low corpus luteum numbers. Notably, the toxic effects of the RT inhibitor lamivudine on the structure and function of the reproductive organs, the preimplantation development as well as the reproductive outcome reveal the fundamental contribution of RT, either of telomerase or retrotransposable origin, on cell growth, proliferation and differentiation control. Future studies examining consequent mice generations are needed to elucidate the negative effects of lamivudine treatment on the offspring. These studies will enrich our knowledge on the mechanism by which RT controls critical reproductive events like gametogenesis and mouse preimplantation development in mammals.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

C.K., L.L. and I.G. conceived and designed the study; C.K., L.L., A.P., P.P., E.M., T.L., K. G., I. B., G.V., S.M., A.B. conducted the experiments; C.K. and LL. analyzed the data; C.K., L.L. and I.G. wrote the paper; C.K., L.L., A.B., and I.G. discussed the manuscript and provided important feedback; I.G. supervised the project.

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