Background: HIV-infected women under highly active antiretroviral therapy (HAART) undergoing in vitro fertilization (IVF) have a lower pregnancy rate than non-infected controls, which depends on oocyte-related factors. We hypothesized that mitochondrial toxicity caused by antiretrovirals could be the underlying mechanism of such disturbance.

Methods: We have studied 16 and 19 frozen-thawed oocytes obtained after oocyte retrieval IVF cycles from 8 and 14 infertile HIV-infected and uninfected women, respectively, matched by age. At inclusion, HIV-positive women had been infected for >13 years and had received HAART for >9 years, including at least one nucleoside reverse transcriptase inhibitor. All of them had undetectable HIV viral load and a good immunological status.

Mitochondrial DNA (mtDNA) content was determined by quantitative real-time PCR in each individual oocyte.

Results: HIV-infected infertile women on HAART showed significant oocyte mtDNA depletion when compared with uninfected controls (32% mtDNA decrease, \(P < 0.05\)). This oocyte mtDNA depletion was even greater on those HIV-infected women who failed to become pregnant when compared with controls (39% mtDNA decrease, \(P = 0.03\)). No significant correlation was found between mtDNA oocyte content and cumulative doses of antiretrovirals or the immunological status of HIV patients.

Conclusions: Oocytes from infertile HIV-infected HAART-treated women show decreased mtDNA content, and this could explain their poor reproductive outcome.

Introduction

The ability of highly active antiretroviral therapy (HAART) to decrease viral load to undetectable levels and to increase CD4+ T-lymphocyte levels has drastically reduced mortality and morbidity among HIV-infected patients [1,2]. As a consequence, HIV infection has become a chronic disease.

Many adverse effects have been described and associated with long-term use of antiretrovirals. Most of them (miopathy, lipoatrophy, lactic acidosis, polineuropathy or pancreatitis) have been related to mitochondrial toxicity caused by nucleoside reverse transcriptase inhibitors (NRTIs) [3–7].

The main underlying mechanism described for mitochondrial toxicity is the ability of NRTIs to inhibit mitochondrial DNA (mtDNA) synthesis both by direct inhibition of the human DNA polymerase-\(\gamma\) (the only polymerase responsible for mtDNA replication) and by acting as chain terminators of the growing DNA strand. Consequently, NRTIs lead to the generation of abnormal mitochondria, with decreased numbers of mtDNA molecules per organelle (depletion), as well as an increase of mutations (deletions and/or point mutations) in the mtDNA genome [8–11]. mtDNA is a 16.6 kb double-stranded molecule that only encodes for 13 proteins of the mitochondrial respiratory chain associated with the oxidative phosphorylation process, two mitochondrial ribosomal RNA and 22 mitochondrial transfer RNA. In this context, a depletion of the mtDNA levels could lead to mitochondrial dysfunction and energetic cell impairment.

According to epidemiological data, HIV-infected women have a lower spontaneous fertility rate than uninfected women [12–14]. Recently, we reported the same observation when these women underwent in vitro...
fertilization (IVF) with their own oocytes. Furthermore, no significant reduction in the pregnancy rate was found when healthy donated oocytes were used. These results suggest that the reduced pregnancy rate observed among HIV-infected women on HAART could be attributed to the oocyte [15]. Although the underlying mechanism of this decreased fertility is unknown, it has been shown that sufficient energy production from mitochondria seems to be relevant in oocyte viability and in the development of embryos. Any mitochondrial defect in oocytes could eventually lead to cell dysfunction and infertility. In this sense, it is important to note that, a priori, the toxic effects of NRTIs can affect any cell containing mitochondria because they have not been specifically designed to enter a particular cellular type and that oocytes are post-mitotic cells with no ability to remove damaged mtDNA. We hypothesized that mtDNA content would be depleted in oocytes of infertile HIV-infected women under HAART treatment.

Methods

A total of eight HIV-infected women all undergoing HAART treatment and 14 controls (non-infected women) undergoing IVF for infertility treatment at Clinica Eugin (Barcelona, Spain), matched by age and IVF indication, were included. All women provided informed consent to participate in the study. The duration and number of previous ART cycles, and total dose of follicle stimulating hormone (FSH) for ovarian hyperstimulation, were similar in both groups.

A total of 16 non-suitable oocytes from eight HIV-infected women and 19 non-suitable oocytes from 14 HIV-negative women were obtained after ovarian hyperstimulation performed using recombinant FSH or human menopausal gonadotropin. Hypophysary suppression was obtained using either gonadotropin releasing hormone agonists or antagonists in a short stimulation protocol. Oocytes were recovered 36 h after the administration of recombinant human chorionic gonadotropin hormone.

During an IVF cycle, a proportion of the retrieved oocytes are immature, either during metaphase I (MI; absence of both a germinal vesicle and a first polar body) or at the germinal vesicle stage. On day 1 after sperm insemination, a proportion of metaphase II (mature) oocytes are non-fertilized (NF). These types of oocytes are defined as unsuitable for assisted reproduction. Therefore, this study did not limit the likelihood of achieving a pregnancy. All women from both groups had viable embryos, which were transferred.

Human oocytes can only be assessed during an IVF cycle. Given that mature oocytes are obtained for fertility purposes, the assessment of non-suitable oocytes and NF oocytes from the same cycle from both infected and uninfected women is the best model available, although it has some limitations. Nevertheless, this is currently the only ethically acceptable approach.

Oocytes were obtained after a standard IVF cycle (by the use of hormonal-stimulated poliovulation and ultrasound-guided oocyte retrieval) and treated with hyaluronidase to remove cumulus cells. Mature oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and observation of non-fertilization was performed on day 1 (18–20 h after ICSI). Selected oocytes were individually rinsed in 0.5 ml of PBS 1× (phosphate-buffered saline solution without Ca2+ and Mg2+; Dulbecco's, Irvine-Scientific, Santa Ana, CA, USA) and placed into a DNase/RNase-free cryotube that was frozen in liquid nitrogen.

DNA isolation and mtDNA quantification

Lysis of each individual oocyte was done in the appropriate buffer containing 125 µl of proteinase K solution (2 mg/ml proteinase K; Roche Diagnostics, GmbH, Mannheim, Germany; SDS 1% and 2 mM EDTA pH 8.0), 50 µl of SDS 10% and 750 µl of lysis solution (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EDTA pH 8.0) and incubated for 60 min at 55°C and for 10 min at 100°C. After lysis, we added to each sample 1 µl of pellet paint (Pellet Paint® Co-Precipitant, Novagen, Merck KgaA, Darmstadt, Germany) and 10 µg of glycogen (Roche Applied Science, Mannheim, Germany) as carriers. Total DNA from each sample was isolated by standard phenol-chloroform procedures adapted to microvolumes (1 volume DNA solution:1 volume phenol:chloroform:isoamyl alcohol 25:24:1; Sigma–Aldrich, Inc., St Louis, MO, USA) and then precipitated with 0.1 volumes of 3 M sodium acetate pH 5.3 and 2.5 volumes of absolute ethanol previously kept at -20°C. The pellet of DNA was dissolved in 40 µl of TE 10:1 buffer (10 mM Tris, 1 mM EDTA pH 7.5) and homogenized at 37°C for 2 h. For each DNA extract, the highly conserved mitochondrial ND2 gene levels were measured by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany) in duplicate using the sequences of the primers and the conditions of the PCR reaction previously reported [16]. The results for the mtDNA content were expressed as picograms (pg) of the ND2 gene. The mtDNA content of controls was considered to be 100%.

Statistical analysis

Clinical and epidemiological characteristics of all women included in the study were expressed as means ±SD (quantitative data) and percentages (qualitative data). One-way ANOVA was used to detect significant differences between groups. When ANOVA was significant and comparisons
included three groups, we used the Bonferroni post-hoc analysis to detect which pair of groups showed significant differences. Linear regression analysis was performed to establish any association among mtDNA content and cumulated doses of antiretrovirals or immunovirological parameters. In all cases, P-values <0.05 were considered statistically significant.

Results

Clinical and epidemiological characteristics of HIV-positive infertile women and controls included in the study are detailed in Table 1. The mean ±SD age was 36 ±2 years in the HIV group and 37 ±4 years in the control group. At the time of the IVF cycle, all HIV-infected women had undetectable HIV viral load and a good immunological status. The mean time after HIV diagnosis was >13 years (153 ±57 months). All patients were on HAART (mean duration 101 ±58 months), which included NRTIs in all cases. Infertile HIV-infected women on HAART had 32% lower oocyte mtDNA content than infertile controls (92 ±51 versus 136 ±71 pg, respectively, P<0.05; Figure 1A).

When mtDNA content was analysed and stratified by the type of oocytes (MI or NF), differences between infected and uninfected women were no longer statistically significant. Nevertheless, as shown in Figure 1B, a consistent trend persisted in the two types of oocytes of infected patients, with 27% depletion for MI oocytes and 36% depletion for NF oocytes when compared with controls (94 ±52 versus 129 ±64 pg for MI oocytes; P=0.24 and 90 ±53 versus 140 ±77 for NF oocytes; P=0.15). Lack of statistical differences could be attributable to the small number of oocytes analysed in each subgroup.

Levels of mtDNA were evaluated according to pregnancy outcome of the same IVF cycle. Pregnancy was established if at least one intrauterine sac was revealed by ultrasonography approximately 5 weeks after embryo transfer.

Pregnancy rate per cycle was 25% (4 out of a total of 16 oocytes) among infertile HIV-infected women on HAART and 37% (7 out of a total of 19 oocytes) in the control group. Among HIV-infected women, oocytes from a non-pregnancy had 30% depletion of the mtDNA content when compared with oocytes from a pregnancy cycle (n=12, 83 ±48 versus n=4, 119 ±57 pg, respectively; P=0.237). When compared with the control group (n=19, 100%), oocytes from a non-pregnancy (n=12) showed a 39% decrease in mtDNA levels (P=0.03), whereas oocytes from a pregnancy cycle (n=4) only showed a 13% decrease in mtDNA content (P=0.653). In addition, when comparing oocytes from a non-pregnancy from HIV-infected women with those of uninfected controls, the former showed a 48% decrease in mtDNA (83 ±48 versus 161 ±70 pg, respectively, P=0.004; Figure 2A). However, no differences were found when comparing HIV-positive and HIV-negative oocytes from a successful pregnancy (119 ±57 versus 94 ±53 pg, respectively; P=0.4; Figure 2B).

No significant correlation was found between oocytes mtDNA content and cumulated antiretroviral doses or the immunovirological status of HIV-patients (data not shown).

Discussion

The oocyte is the largest human cell (300x bigger on average than other somatic cells) and contains a large amount of mitochondria that represent at least 23% of the ooplasm [17].

Mitochondria are double-membrane intracellular organelles and the main source of the high-energy phosphate molecule adenosine triphosphate, which is essential for all active intracellular processes [18]. Oocytes are packed with mitochondria, and disorders of mitochondrial function could cause reproductive failure. mtDNA copy number per mature human oocyte is about 100,000–600,000 molecules compared with 500–10,000 molecules for somatic cells [19,20]. Furthermore, the oocyte contains approximately one mtDNA molecule per mitochondrion to avoid heteroplasmic segregation through the maternal lineage [21] and differs from the 2–10 mtDNA copies in human somatic mitochondria [17]. This fact makes oocyte mitochondria especially vulnerable to mtDNA depletion and oocyte especially sensitive to mtDNA depleting factors (drugs, toxins and infections).

Mitochondrial dysfunction has been associated with reproductive outcome because their functionalism influences the viability of both sperm and oocytes. Accordingly, low mtDNA content in both male [22]
and female gametes [17,23,24] has been associated with infertility. Mutations in the mtDNA genome have also been described in spermatozoa with declined motility and fertility [25]. Other investigations relate the generation of abnormal sperm mtDNA molecules (with multiple mtDNA deletions) to long-term antiretroviral intake in HIV-infected patients [26]. Moreover, it has been suggested that HIV infection and NRTIs have negative effects on semen parameters (semen volume, percentage of progressive motile spermatozoa, total sperm count, polynuclear cell count, pH and spermatozoa anomalies), compromising male fertility.

In the oocyte, mitochondria contribute to fertilization and embryonic development. Cohen et al. [27] reported that ooplasm transfer (including mitochondria) from a young donor oocyte partially restores the reproductive capacity in oopausic oocytes. Both Reynier et al. [23] and Santos et al. [17] established an association between mtDNA content and the probability of oocyte fertilization. The latter suggested that the mtDNA content could be an oocyte quality and fertility marker. Another study suggested that low mtDNA content was associated with impaired oocyte quality observed in ovarian insufficiency [24]. These studies suggest that mitochondria are crucial to fertilization outcome and embryonic development [27]. Nevertheless, there are no published studies assessing mtDNA levels in oocytes of HIV-infected women.

Epidemiological and clinical data suggest that HIV-infected women on HAART undergoing IVF had lower pregnancy rates than uninfected women (16.2 versus 39.2%) [15]. However, the effect of HIV infection was not observed in women undergoing oocyte donation (36 versus 45.1%).

This is the first study to address the underlying mechanism that could explain a low pregnancy rate in HIV-infected women. Our data suggest that oocytes from infertile HIV-infected women on HAART have decreased mtDNA levels compared with infertile uninfected controls.

Two possible mechanisms could explain the mtDNA depletion found in our study. One of them is related to the secondary effects of antiretrovirals and oocyte characteristics. HAART combinations to treat HIV, specifically those containing NRTIs, could cause mitochondrial toxicity. Any cell that contains mitochondria is susceptible to the toxic effects of NRTIs. Therefore, oocytes might be exposed to cumulated therapeutic doses of these drugs. Oocytes are post-mitotic cells with no ability to eliminate damaged mitochondria and with a high dependence on the oxidative phosphorylation system. Moreover, because oocytes contain a large number of mitochondria with only one molecule of mtDNA per organelle, they are more sensitive to mtDNA depleting factors. In this scenario, oocytes would be especially prone to decrease the mtDNA levels. On the other hand, HIV might induce unspecific mitochondrial damage in other tissues and cause disruption of the mitochondria-mediated apoptotic mechanisms, thereby indirectly depleting oocyte mtDNA levels [28]. We did not find

Figure 1. Oocyte mitochondrial DNA content for HIV-treated women and uninfected controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls (n=19)</th>
<th>HIV-positive (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI and NF oocytes</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>mtDNA content, pg</td>
<td>250</td>
<td>200</td>
</tr>
<tr>
<td>Controls</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>HIV-positive</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>P=0.24</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls (n=7)</th>
<th>HIV-positive (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI oocytes</td>
<td>P=0.15</td>
<td></td>
</tr>
<tr>
<td>mtDNA content, pg</td>
<td>250</td>
<td>200</td>
</tr>
<tr>
<td>Controls</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>HIV-positive</td>
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<tr>
<td>P=0.24</td>
<td>0</td>
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<table>
<thead>
<tr>
<th>Group</th>
<th>Controls (n=12)</th>
<th>HIV-positive (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF oocytes</td>
<td>P=0.05</td>
<td></td>
</tr>
<tr>
<td>mtDNA content, pg</td>
<td>250</td>
<td>200</td>
</tr>
<tr>
<td>Controls</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>HIV-positive</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>P=0.15</td>
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</table>

Oocyte mitochondrial DNA (mtDNA) content, expressed as picograms (pg) of the ND2 gene, for controls (uninfected women) and HIV-positive women (A) when both metaphase I (MI) and non-fertilized (NF) oocytes were analysed together and (B) when analysed according to the type of oocyte (MI or NF).
an association between mtDNA content and the length of antiretroviral therapy and immunovirological status of patients. Therefore, we cannot further demonstrate the contribution of each mechanism. However, we believe that HIV should not have a direct effect on the human oocyte because no receptors for HIV have been described on either the cumulus cells or on the surface of the oocyte.

According to the abovementioned factors, the most plausible hypothesis is that the underlying mechanism that causes oocyte mtDNA depletion and reduced fertility among HIV-infected women on HAART is the antiretroviral therapy. However, we are not able to give a firm conclusion because all HIV-infected women included in our study were on HAART. In this context, it is not possible to determine whether these results are attributable to HIV-infection or to HAART. No data are available on mtDNA content of oocytes from HIV-infected non-HAART-treated women.

Interestingly, among HIV-infected women, mtDNA content in the non-viable oocytes from women that did not become pregnant was lower than that among women who achieved a pregnancy, although differences where not significant (83 ±48 versus 119 ±57 pg, respectively, P=0.237). However, this mtDNA depletion was significant when comparing HIV-infected women who did not become pregnant with uninfected controls (39% depletion, P=0.03). Lack of statistical significance for the first comparison could be attributed to the small number of patients in the subgroups analysed. These results could explain the low pregnancy rate after IVF observed among HIV-infected women.

According to our results, the lower fertility rate observed in IVF cycles among HIV-infected women under antiretroviral therapy might be explained by oocyte mitochondrial impairment that is secondary to mitochondrial toxicity by HAART. However, we cannot rule out the potential effects that HIV could indirectly have on fertilization outcome and on mtDNA content through other unknown biological causes. Further studies are required to investigate this issue.

Acknowledgements

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References


