Effects of combined zidovudine/lopinavir/ritonavir therapy during rat pregnancy: morphological aspects

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Summary

Purpose: To evaluate the morphological aspects in rats subjected to an association of the antiretroviral drugs zidovudine/lopinavir/ritonavir in different doses administered throughout the gestational period. *Materials and Methods:* Forty pregnant rats were randomly allocated into four groups: control (Ctrl) and experimental (Exp1, Exp2, and Exp3), which received zidovudine/lopinavir/ritonavir in the doses of 10/13.3/3.3, 30/39.9/9.9, and 90/119.7/29.7 mg/kg per day from the first to the 20th day of pregnancy, respectively. At term, the animals were euthanized and maternal and fetal organ samples were removed for morphological analysis. *Results:* No major changes were identified in the group treated with the lowest dosing compared with the control. In group Exp2, the authors found hepatocytes with eosinophilic cytoplasm, pyknotic nuclei, and vasodilation. The proximal convoluted tubules of maternal kidneys showed eosinophilic areas and hyperchromatic nuclei, as well as signs of vasodilation. In the group treated with the highest dose (Exp3); the morphological changes in the maternal kidneys and livers were similar and more pronounced than those found in Exp2. The maternal pancreas of groups Exp2 and Exp3 evidenced moderate and progressive signs of tissue damage. The morphological features of all fetal livers, kidneys, and pancreases were normal. *Conclusion:* High doses of zidovudine/lopinavir/ritonavir association during the entire rat pregnancy period can cause definite morphological changes in maternal liver, kidneys, and pancreas. On the other hand, the corresponding fetal organs were not affected.

Key words: Zidovudine; Lopinavir; Ritonavir; Toxicology; Pregnancy; Rats.

Introduction

Due to the worldwide situation of human immunodeficiency virus (HIV) infection, taking into consideration epidemiological and clinical aspects, there is an urgent need to deepen our knowledge about the obstetrical challenges related to HIV-infected women [1, 2].

In 1994, under a grant from the National Institute of Allergy and Infectious Diseases of the United States of America, the Aids Clinical Trial Group (ACTG) performed studies and found that zidovudine (AZT) reduced the perinatal transmission of HIV to newborns of infected women by a two-third rate [3, 4]. Based on this outcome, zidovudine became the leading monotherapic drug for prevention of perinatal HIV transmission. The use of this substance in different countries has resulted in lower levels of vertical transmission of the virus. Later, experiments (ACTG 175) showed a desirable effect of zidovudine on pregnant women with cell counts of CD4 < 200 cells/mm^3 (i.e., strongly immunodepressed), as well as on women taking AZT before pregnancy [5]. Between 1994 and 1997, other blockers of protease activity were discovered, such as ritonavir and lopinavir. Though side-effects of associations of these drugs can be worse than those due to each drug alone, their antiviral effects are significantly superior. The association results in a significant reduction of viral load in plasma, sometimes to undetectable levels of the virus [6].

One of the several treatment protocols of highly active antiretroviral therapy (HAART) usually includes one nucleoside analog (DNA chain terminator), one protease inhibitor, and either a second nucleoside analog ("nuke") or a non-nucleoside reverse transcription inhibitor [7]. Though the association of antiretroviral drugs has been shown to be largely safe for the concept [8], the concomitant use of multiple drugs can cause alterations in their pharmacokinetics [9], with unpredictable results on the maternal and/or fetal compartments.

Recently, the present laboratory showed that ritonavir administered to pregnant rats during the entire period of gestation causes definite deleterious effects on fertility and a high maternal mortality rate coexisting with a 100% fetal survival rate, thus suggesting an important compartmentation of the drug [10]. Cunha *et al.* [11] showed that the administration of a combination of lopinavir plus ritonavir to pregnant rats can cause morphological as well as functional changes in maternal and fetal livers and kidneys, and supratherapeutic doses can be toxic to the animals. Although one cannot extrapolate animal drug effects to human beings, an acceptable first approach to the understanding of human drug effects is their study in animal models.

In the present laboratory, the authors aimed to search for some pharmacokinetic changes of the antiretroviral drugs

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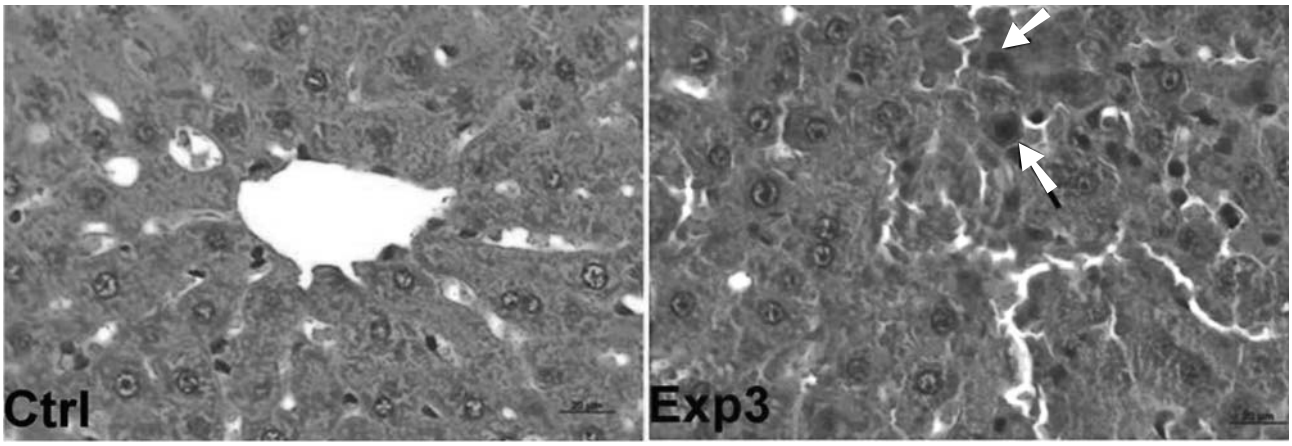

Figure 1. — Photomicrographs of typical histological sections of rat livers from the control groups (Ctrl) and treated with zidovudine/lopinavir/ritonavir (Exp3, 90/119.7/29.9 mg/kg) throughout gestation. Hepatocytes with eosinophilic cytoplasm and heterocromatic nuclei (arrows) can be observed. x 200, H&E staining.

that could provoke adverse effects on the liver, pancreas, and kidneys of pregnant rats and their fetuses. Since few trials have been conducted on the effect of antiretroviral drugs on rat pregnancy, the authors decided to test doses of zidovudine/lopinavir/ritonavir in association, corresponding to one, three, and nine times the human therapeutic doses. These same doses have previously been investigated in an experimental model study that appraised the effects on mothers and litters.

Materials and Methods

Female Wistar rats *(rattus norvegicus albinus)* of the EPM-1 variant, weighing approximately 200 g each, were provided by the Center for Development of Experimental Models of São Paulo Federal University (UNIFESP). The study was approved by the local animal care committee (Report 0402/09) and followed the guidelines proposed by the Canadian Council on Animal Care [12].

The animals were kept in plastic cages under controlled room temperature (22°C) and artificial light by fluorescent lamps with a constant day/night cycle (lights on 07:00-19:00), with free access to pelleted rat food and tap water. After a seven-day period of adaptation, the animals were allowed to mate in the proportion of one healthy male to three females during two hours. The immediate 24-hour period after mating was taken as day (0) of pregnancy if spermatozoids were detected in vaginal smears [13]. Forty pregnant rats were randomly divided into four groups. The control group (Ctrl) received distilled water and the experimental groups (Exp1, Exp2, and Exp3) received zidovudine/lopinavir/ritonavir in the corresponding doses of 10/13.3/3.3, 30/39.9/9.9, and 90/119,7/29.7 mg/kg/day by gavage from the first to the 20th day of pregnancy [14, 15].

At term, the animals were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) by intraperitoneal route. Upon laparotomy, four ml of maternal blood was taken directly from the ventricular chambers for further biochemical determinations: aspartate transaminase (AST) and alanine aminotransferase (ALT), blood urea nitrogen (BUN), lipase and creatinine, cholesterol activity, and glucose. Fetuses were extracted upon a longitudinal uterine incision. Maternal and fetal samples of livers and kidneys and maternal pancreas were taken and fixed in buffered ten percent formaldehyde for further routine processing, hematoxylin-eosin (H&E) staining, and light microscopy study.

Statistical analyses

The results were analyzed by one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons test. Contingency tables were prepared and the Chi-square test was performed to analyze the death rate among the groups. The 2.01 GraphPad InStat software was used for this purpose. The differences were considered statistically significant when $p < 0.05$.

Results and Discussion

The light-microscopic appearance of the maternal livers in the Ctrl and Exp1 groups was essentially undistinguishable. Structures in samples from group Exp2 appeared very similar to those from the Ctrl and Exp1 groups, but congestion could be seen. The same but even more marked alterations were seen in the Exp3 group. There were areas containing a great number of hepatocytes with eosinophilic cytoplasm and heterochromatic nuclei, and extensive congestion (Figure 1).

No significant differences were noticed regarding the values of ALT and lipase activity among the groups (Table 1). Since this activity is taken as an index of the functional status of the hepatic and pancreatic tissues, it seems that the side-effects of the treatment with the transcriptase reverse inhibitor zidovudine and the protease inhibitors ritonavir and lopinavir, even at the highest dosing (Exp3), did not significantly impair the functional capacity of the pregnant rat liver. On the other hand, the levels of AST were significantly higher in group Exp3 ($p < 0.05$) than in the Ctrl and Exp1 groups. Since the AST activity is much less related to liver function than to the functional status of other organs and tissues, the observed alterations could well be due to some muscle alterations. In fact, the association of lopinavir with ritonavir has been reported to cause muscular disorders [14]. Moreover, it has been reported that reverse transcriptase inhibitors, such as

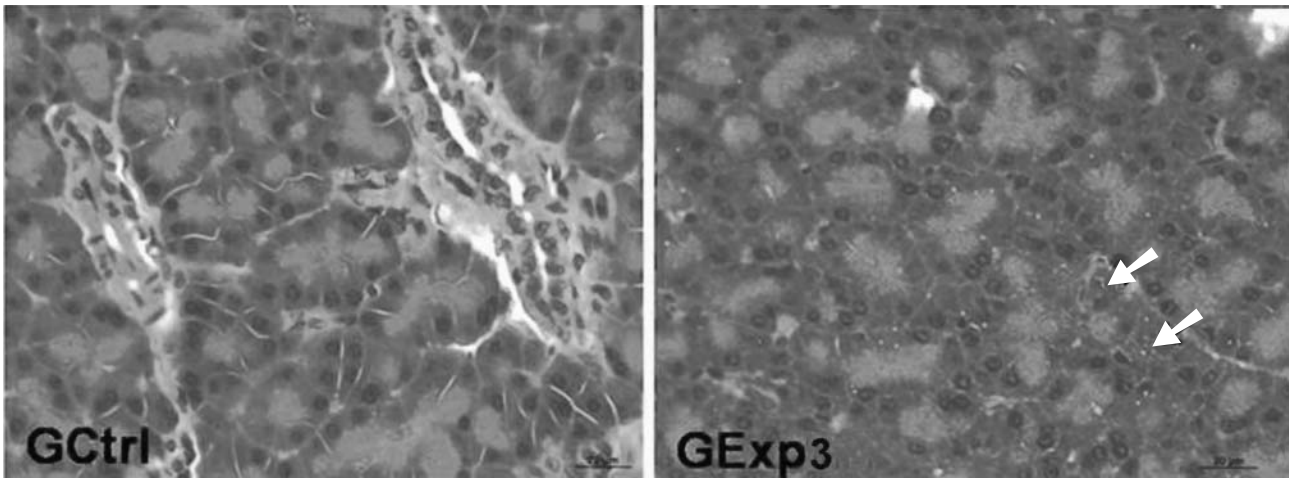

Figure 2. — Photomicrographs of typical histological sections of a rat pancreas from the control group (Ctrl) and treated with zidovudine/lopinavir/ritonavir (Exp3, 90/119.7/29.9 mg/kg) throughout gestation. The Exp3 group shows fat infiltration (arrows). x 200, H&E staining.

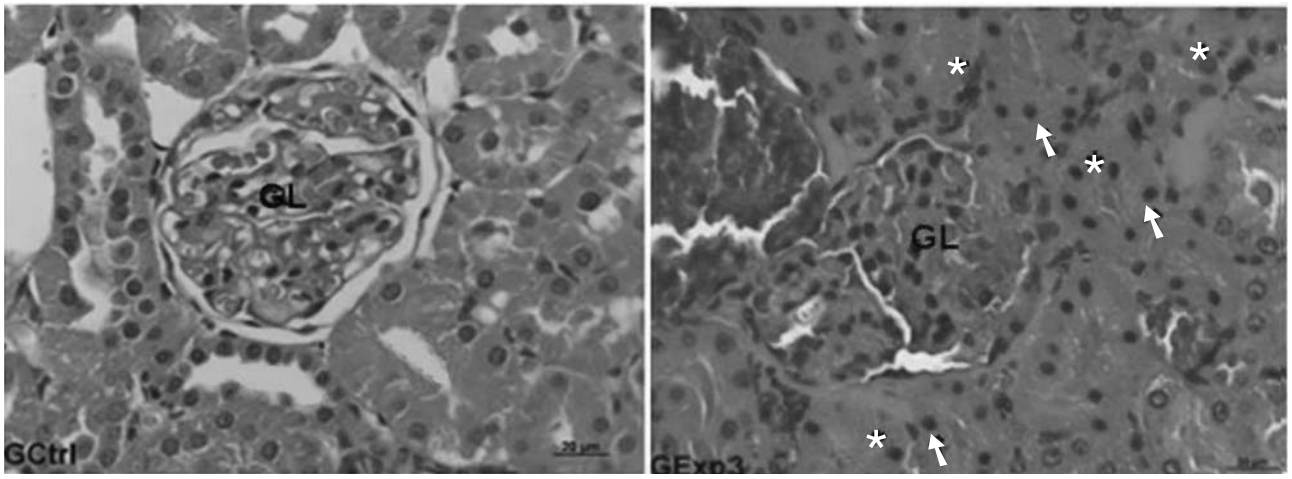

Figure 3. — Photomicrographs of typical histological section of a maternal kidney from the control group (Ctrl) and treated with zidovudine/lopinavir/ritonavir (Exp3, 90/119.7/29.9 mg/kg) throughout gestation. Observe glomerulus (GL) and proximal convoluted tubules show eosinophilic areas (*) and hyperchromatic nuclei (arrows). H&E staining, x 200.

zidovudine, bind to mitochondrial DNA polymerase gamma, leading to mitochondrial dysfunction and consequently myopathy, myelosuppression, pancreatitis, peripheral neuropathy, and hepatic steatosis [16].

The authors found no significant differences in cholesterol and triglycerides in the four groups. It should be mentioned that a study of lopinavir/ritonavir for 24 weeks showed a rise in triglycerides and total cholesterol, and body fat gain, but these changes were not related to the plasma level of lopinavir [17]. Another study evaluating 22 patients who received lopinavir-ritonavir showed severe dyslipidemia when lopinavir plasma concentrations were above eight mg/ml [18]. It should be mentioned that some lipid droplets were found in the maternal pancreas in group Exp3 (Figure 2).

In the present experiment, the authors noticed reduced serum glucose levels in the Exp3 group compared to the other groups. This could be explained by the fact that combination therapy (HAART) can lead to glucose intolerance, similar to that seen in type I diabetes, suggesting a deleterious effect on the pancreas whereby the conversion of proinsulin into insulin is inhibited due to the activity of protease inhibitors [19]. The facilitated transport of glucose is an energy-independent system which has the primary function of mediating the exchange of glucose between blood and cell cytoplasm, forming a selective pattern among the three major pools of glucose, i.e., the brain, extracellular fluid, and cytoplasm. There are at least seven glucose transporters in mammals. GLUT 1, also called glucose transporter type HepG2, expressed in adult hepatocytes and fetal tissue, is present in erythrocytes. The reduction in blood glucose evokes an increase in GLUT 1, resulting in an increased flow of glucose through the blood-brain barrier, as well as being responsible for the supply of glucose to the placenta. GLUT 3 is the largest carrier in the placenta of rats. It is located in

Table 1. — *Effects of treatment with a zidovudine/lopinavir/ritonavir combination during the entire period of rat gestation on AST and ALT activities, and on the levels of BUN and creatinine, lipase, glucose, cholesterol, and triglicerides in maternal blood at term.*

Groups	AST (mU/ml)	ALT (mU/ml)	BUN (mg/100 ml)	Creatinine (mg/100 ml)	Cholesterol (mg/dl)	Triglicerides (mg/dl)	Glucose (mg/dl)	Lipase (UI)
Ctrl	92.8 ± 14.2	55.6 ± 4.6	50.6 ± 1.4	0.35 ± 0.02	118.4 ± 7.06	308.5 ± 0.01	98.2 ± 10.11	21.2 ± 5.31
Exp1	70.2 ± 22.5	52.1 ± 5.4	49.9 ± 3.6	0.34 ± 0.01	117.1 ± 6.02	410.2 ± 0.01	104.8 ± 5.01	25.7 ± 3.07
Exp2	100.6 ± 12.1	54.0 ± 4.2	50.9 ± 3.1	0.35 ± 0.04	110.4 ± 8.01	290,3 ± 0.01	100.9 ± 4.02	19.8 ± 4.21
Exp3	138.8 ± 10.4*	60.9 ± 5.6	58.7 ± 1.2*	0.32 ± 0.03	108.7 ± 8.02	346,9 ± 0.01	85.8 ± 12.15	27.4 ± 5.21

Values are mean ± standard deviation. Groups of pregnant animals (n = 10 for each group) were treated once a day by gavage with distilled water (Ctrl) or zidovudine/lopinavir/ritonavir (Exp1, 10/13.3/3.3 mg/kg; Exp2, 30/39.3/9.9 mg/kg; Exp3, 90/119.7/29.9 mg/kg) throughout gestation (*$p < 0.05$).

the syncytiotrophoblast and is involved in the transfer of glucose to the embryo; it has a high affinity for glucose [20]. A study conducted in 15 HIV-positive patients on the use of lopinavir/ritonavir with hyperinsulinemia and/or dyslipidemia, showed that substitution of lopinavir/ritonavir with atazanavir/ritonavir increased uptake of glucose by muscles in vivo and decreased fasting glucose [21]. These findings are consistent with inhibition of GLUT 4 by lopinavir and ritonavir [22]. It is believed that this finding is due to malnutrition caused by gastrointestinal problems (nausea, poor appetite, and diarrhea), which are adverse effects of lopinavir/ritonavir. A study evaluating the effects of protease inhibitors on the metabolism of glucose found that long-term exposure to protease inhibitors not only induced peripheral insulin resistance, but also affected insulin secretion stimulated by glucose in pancreaticβ cells, resulting hyperglycemia [23]. The authors found the opposite in the Exp3 group.

Significant differences were observed regarding the maternal kidneys of the Ctrl group and the other groups under microscopic examination. The gross structural appearance of the kidneys of groups Exp1 and Exp2 were very similar to those and the proximal convoluted tubules showed eosinophilic areas and hyperchromatic nuclei; some vasodilation could also be seen. The kidneys from group Exp3 were structurally similar to those from the other groups. However, more severe alterations of the proximal tubules were seen and there were conspicuous glomerular damages and more extensive vasodilation. These morphological changes suggest that, although three to four percent of the elimination of the tested drugs occurs through the kidneys, there were just mild deleterious renal effects.

Since the circulating plasma levels of creatinine did not increase and BUN increased in the Exp3 group (Table 1), it is conceivable that the functional capacity of the maternal kidney was able to overcome the effects of the drugs in the doses studied here. The mean values of these metabolites (BUN) were significantly higher in the group Exp3 than those observed in the control animals (Table 1). A possible explanation for this result is drug-induced alteration of the morphology and intrarenal hemodynamics. In fact, since the vasodilation seen in the treated groups (Figure 3) could also occur at the afferent arteriole level, increased serum concentrations of creatinine would have been eliminated through the urine, thus resulting in reduced circulating concentrations of creatinine, but no BUN. The concentration of urea has a higher positivity rate than creatinine, which often can be indicative of some potential renal adverse effects.

Regarding the fetal organs, the morphological appearance in the control group showed proper development of the organs for age. The experimental groups did not present any developmental abnormalities. The passage of various compounds through the placental barrier is blocked or significantly impaired by the placental p-glycoprotein [24]. Other barriers in which P-gps are importantly involved are the blood-brain, the blood-nerve, the blood-testis, and the intestinal barriers [25].

Conclusion

In conclusion, pregnant rats treated during the entire gestation period with doses of zidovudine plus lopinavir plus ritonavir up to nine times those indicated for humans caused dose-dependent focal morphological changes in maternal liver and kidneys. However, the morphological alterations were not paralleled by systemic biochemical changes, which could be interpreted as specific damages to these organs. Fetal livers and kidneys from animals which had been treated with the highest drug doses did not show morphological alterations.

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