General Section

Chronic action of association of zidovudine, lamivudine and ritonavir on pregnant rats. A biologic assay

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Summary

Purpose: To evaluate at term the effects of a highly active antiretroviral (HAAR) drug association administered during the entire period of rat pregnancy. *Methods:* Three groups (n = 10 each) of adult pregnant rats were treated with an oral solution of HAAR (Exp 1 = 10/5/20 mg/kg b.w.; Exp 2 = 30/15/60 mg/kg b.w.; Exp 3 = 90/45/180 mg/kg b.w.) from day "0" up to the 20th day of pregnancy. A fourth group served as a control. At term (20th day) the rats were killed under deep anesthesia and the number of implantations, resorptions, living fetuses, placentae and intrauterine deaths were recorded. *Results:* The highest HAAR doses caused lower maternal weight gain, lower litter weights, and lower placental weights compared to the control group. *Conclusions:* HAAR during the entire period of rat pregnancy can reduce maternal body weight gain and lower term placental weight.

Key words: Rat; Pregnancy; Zidovudine; Lamivudine; Ritonavir.

Introduction

Acquired immunodeficiency syndrome (AIDS), although first described during the 1980s, is still at the center of global investigations [1]. According to the *Joint United Nations Program on HIV/AIDS (UNAIDS)* estimates, there are around 33 million people living with HIV. Of these, 22.5 million are in the Sub-Saharan Africa. Among the 33 million people infected, 15.5 million are women. According to estimates obtained through the joint data from UNAIDS, the *World Health Organization (WHO*) and the United Nations Fund for Children (UNICEF) and countries which report their metrics, it appears that the antiretroviral coverage for HIV-positive pregnant women for vertical transmission prevention went from 9% in 2004 to 33% in 2007 [2].

Viral transmission from mother to child is responsible for most of the AIDS cases in children - which can happen in three periods: during pregnancy (transplacental), during delivery, and through breastfeeding. The HIV vertical transmission rate, when all prophylactic interventions are used, can affect about 25% of the newborns from HIV+ mothers, and it can be reduced to levels as low as 1-2% by following proper measures during prenatal care, delivery and puerperium [3]. These interventions include: the use of antiretroviral agents starting at the 14th week of gestation; the use of injections of zidovudine (AZT) during delivery; C-section delivery when indicated; oral AZT for exposed newborns from birth through 42 days of life and breastfeeding inhibition associated with the use of infant milk formulas up to six months of age [3]. Today we have five groups of antiretroviral agents: nucleoside analog reverse transcriptase inhibitors (NARTIs); non-nucleoside analog reverse transcriptase inhibitors (NNARTIs); protease inhibitors (PI); fusion inhibitors (FI): enfuvirtide (ENF or T20), maraviroc and integrase inhibitors - (raltegravir) not used in pregnant women [4].

The objective reduction in HIV vertical transmission with the use of antiretroviral drugs is seen as one of the most noteworthy progresses among those who investigate such topic. The combination of three or more antiretroviral agents, recently called HAART (*highly active antiretroviral therapy)* is recommended during pregnancy, and moreover many women become pregnant when already under treatment. Nonetheless, despite such remarks it should be stressed that studies which assess the safety of such drugs during pregnancy are necessary, especially regarding their toxicity and teratogenic potential.

The use of a drug during pregnancy demands the investigation of its effects on the fetus and the newborn, especially regarding teratogenicity, mutagenicity and carcinogenesis depending on the pharmacokinetics and drug transplacental transfer toxicity. Thus, the effect of a certain drug on the fetus will depend on the dose the mother ingested, gestational age at which the fetal is exposed, exposure duration, interaction with other drugs and other factors such as maternal and fetal genetics [5].

Aiming to clarify the combined action of antiretroviral agents during pregnancy, our group decided to study the chronic use of zidovudine associated with lamivudine and ritonavir on the pregnancy of albino rats in a biological assay.

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Materials and Methods

Wistar female rats (*Rattus norvegicus albinus*) of the EPM-1 variant, with approximately 250 g of body weight provided by the Center for the Development of Experimental Models (CEDEME) of the Federal University of São Paulo – Escola Paulista de Medicina (UNIFESP-EPM) were used throughout the study. Experiments were approved (Report no. 0749/07) by the local Animal Care Committee, following guidelines which comply with those of the Canadian Council on Animal Care [6].

The animals were kept in plastic cages under controlled room temperature set at 22ºC and artificial light by fluorescent lamps with a photoperiod of 12 h (lights on at 7 a.m.), with free access to pelleted Purina rat diet and tap water.

After a 7-day period of adaptation, the animals were mated in the proportion of one male to three females during 2 h. The immediate 24-h period after mating was taken as day 0 of pregnancy if spermatozoids were detected in vaginal smears [7]. Forty pregnant rats were then distributed at random into four animal groups, as follows. Control (n = 10) were rats treated daily with 0.5 ml of propyleneglycol by the oral route (drug vehicle controls). Exp 1 (n = 10) were rats treated with an association of oral of zidovudine/lamivudine/ritonavir corresponding to a daily dose of 10 mg/kg zidovudine plus 5 mg/kg of lamivudine plus 20 mg/kg of ritonavir (zidovudine, lamivudine, GlaxoSmithKline Laboratories, London, plus ritonavir, Abbott Laboratories, Chicago, IL). Exp 2 (n = 10) were similarly scheduled rats treated daily with 30, 15 and 60 mg/kg of zidovudine/lamivudine/ritonavir, respectively. Finally, Exp 3 (n = 104) rats were treated with 90, 45 and 180 mg/kg of zidovudine/lamivudine/ritonavir respectively. Vehicle and drugs were administered by gavage, once daily, in a final volume of 0.5 ml, starting at day '0' and extended until the term of pregnancy.

Body weights were recorded for all animals on day 0, 7, 14 and 20 of pregnancy and expressed as percentuals of body weight gain.

At term (20th day), the animals were weighed and anesthetized with a mixture of xylazine (20 mg/kg) and ketamine (100 mg/kg) by the intraperitoneal route. Upon wide open laparotomy and hysterotomy, the following parameters were recorded: fetal and placental weights, number of implantations, number of reabsorptions, number of living and dead fetuses. The fetuses were closely examined under a stereoscope microscope for gross external malformations (limb shortening, bifid spine, cleft lip, cleft palate and hypospadia).

Whenever appropriate the data are expressed as mean ± SEM; the results were subjected to ANOVA and further analyzed by the Kruskal-Wallis multiple comparisons test. Contingency tables and chi-square tests were used to analyze the death rates. The significance level was set at 5%.

Results and Discussion

Since maternal weight gain depends on the initial and final weight of the rats, we considered the most reliable weight percentage difference in order to analyze such data, because there was a weight variation in the beginning of the experiment among the different groups investigated. By analyzing Figure 1, it can be noted that percentage-wise, groups Exp 1 and Exp 2 gained more weight than Group Exp 3 by the 14th day of gestation, the time during which fetal weight has very little impact on maternal weight. It can then be concluded that, with the treatment dose and up to three times such dose, there was

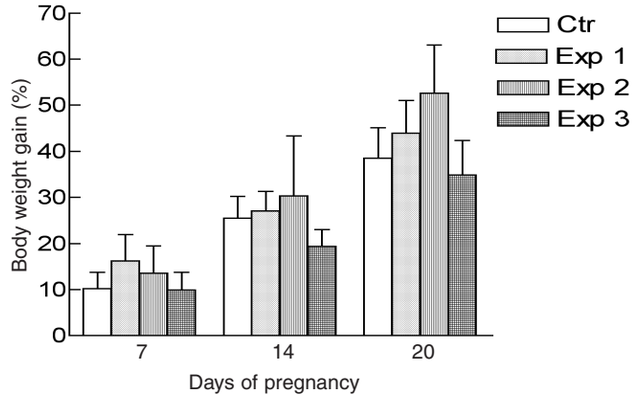

Figure 1. – Profiles of body weight gain of pregnant rats expressed as % of the starting weight (at day 0 of pregnancy). The rats (n = 10 throughout) were treated as follows. Control (Ctr) = drug vehicle (propyleneglycol); experimental animals were treated once a day with association zidovudine/lamivudine/ritonavir by gavage during the entire period of gestation: Exp 1 = 10/5/20 mg/kg, Exp 2 = 30/15/60 mg/kg, and Exp 3 = 90/45/180 mg/kg b.w., respectively. Within every gestational day, the significant differences regarding the corresponding Ctr values are as indicated: at the 14th day, *Exp 3 < Ctr = Exp 1 = Exp 2 ($p < 0.05$); at the 20th day, †Exp 2 > Ctr = Exp 3 ($p = 0.001$); ‡Exp 3 < Exp 1 ($p = 0.001$).

a percentage weight gain beyond what is normal for pregnancy, which could be explained by the effect zidovudine and ritonavir have on lipid (hyperlipidemia) and carbohydrate (hyperglycemia) metabolism, together with the lipodystrophy these rats have [8-11].

Lipodystrophy syndrome is characterized by an abnormal redistribution of body fat. Lipodystrophy includes: build up of central fat concentrating in the breasts, abdomen, subcutaneous cell tissue and the posterior neck region; and lipoatrophy: reduction in the peripheral subcutaneous cell tissue (face, gluteus, upper and lower limbs), producing a pseudo-athletic appearance, with prominent muscles and vessels. Such fat redistribution syndrome may also be associated with metabolic changes such as dyslipidemias, insulin resistance, lactic acidosis, hypogonadism and osteoporosis [12].

It was first believed that protease inhibitors were the causal agents. Later on, lipodystrophy was described in patients not using PI, making it clear that its genesis is multifactorial, including genetic factors, age, gender, duration of exposure to antiretroviral agents, metabolic alterations, and CD4 nadir at treatment onset, among others. The lipodystrophy etiology seems to be related to mitochondrial toxicity associated with reverse transcriptase inhibitors, adipocytes apoptosis associated with PI use, TNF-alpha deregulation, PI-related cytochrome p450 inhibition, local HIV effect on cortisol production and alterations in other steroid hormones [12].

Analyzing the percentage weight gain on the 20th day of pregnancy, it was noted that there was also higher and more significant weight gain in experimental groups Exp 1

and Exp 2, when compared to the control group and experimental group Exp 3 (Figure 1). Such findings suggest a possible toxic effect on rat weight gain when higher doses of drugs were used (Exp 3).

When used alone, zidovudine did not change maternal weight gain, even at high doses of 60 and 100 mg/kg per day, as mentioned by Mamede *et al*. [13, 14] and Figueiró Filho *et al*. [15], respectively. In a study carried out by Pontes *et al*. [16], using lamivudine (doses equal to the ones used in this study) alone during the entire albino rat pregnancy, they did not notice significant weight gain among the mothers. However, Figueiró Filho *et al*. [15], using lamivudine ten times the dose normally used in pregnancy in adult Wistar pregnant rats, observed that the mothers whose treatment regimen included this antiretroviral agent (3TC, AZT+3TC and AZT+3TC+NFV) had a significant weight gain, as well as higher fetal weight during birth. Nakamura [17] in a horizontal study comparing the actions of six antiretroviral agents in immunotherapy during the entire pregnancy of albino rats noticed that rats exposed to lamivudine had weight gain reduction on the 20th day when compared to the ones that used stavudine and nelfinavir. Ritonavir, when used alone by Carvalho *et al*. [18], did not influence the weight gain of pregnant rats during the entire pregnancy, except in the group that received nine times the therapeutic dose, which weight gain was numerically lower than the other groups since pregnancy onset; however, it was only statistically significant in relation to the control group on the 20th day. Such weight reduction was attributed to dose-dependent gastrointestinal side-effects as a function of ritonavir exposure duration.

As to the number of implantations, resorptions, fetuses and placentas in the present study, statistically significant differences were not found among the four groups studied (Table 1).

Zidovudine in doses up to 60 mg/kg per day (6 times the therapeutic dose), studied by Mamede *et al*. [13], did not increase the rates of fetal resorption. On the other hand, Figueiró Filho *et al*. [15] studied the action of numerous antiretroviral agents during albino rat pregnancy and found an increase in the resorption rate (26.7%) when they used zidovudine at the dose of 100 mg/kg per day (10 times the treatment dose), suggesting that in high doses the drug does interfere with resorption rates.

Figueiró Filho *et al*. [15] used lamivudine (10 times the therapeutic dose) in adult Wistar pregnant rats and found higher rates of resorption – 26.3%, when used alone, 24.2%, when associated with zidovudine, and 23.5%, when associated with zidovudine and nelfinavir. However, Pontes *et al*. [16] did not find significant differences regarding the number of implantations and resorptions using lamivudine in doses equal to the ones used in our study, thus suggesting that when used alone it does not interfere with rat fecundity.

Carvalho *et al*. [18] used ritonavir alone and found a similar number of implantations, both in the experimental groups studied and in the control group; however, the resorption rate was statistically higher in the Exp 3 group

Table 1. — *Effects of extended administration of the association zidovudine/lamivudine/ritonavir on several indicators of rat pregnancy; Groups are as in the legend to Figure 1. Data are expressed as mean ± SEM. No fetal external malformations were observed (n = number of animals).*

	GROUPS			
	Control (n =10)	Exp 1 (n = 10)	Exp 2 (n = 10)	Exp 3 (n = 10)
No. of fetuses	11.10 ± 2.00	10.00 ± 1.90	8.70 ± 1.80	8.70 ± 3.10
No. of placentae	11.10 ± 2.00	10.00 ± 1.90	8.70 ± 1.80	8.70 ± 3.10
Litter weight (g)	47.00 ± 8.90*	54.50 ± 7.30	42.20 ± 8.70	35.80 ± 14.30
Placenta weights (g)	8.66 ± 2.04	9.65 ± 3.29	8.65 ± 2.40	6.10 ± 2.30
No. of implantations	11.10 ± 2.00	10.00 ± 1.90	8.70 ± 1.80	8.90 ± 2.90
No. of reabsortions	0	0	0	0.20 ± 0.40
Maternal deaths	0	0	0	0
Fetal deaths	0	0	0	0

*$p \leq 0.01$ with regard to Exp 2 and Exp 3.

(36%), which received 180 mg/kg per day (9 times the therapeutic dose) in relation to controls. The study carried out by Fontes *et al*. [19], using zidovudine together with ritonavir, showed results which were similar to ours as far as implantations are concerned. As to the number of resorptions, the result was significantly higher in group Exp 3 ($p < 0.01$) when compared to the control group, causing a resorption rate of about 18%.

These data allow us to think that the influence of zidovudine, lamivudine and ritonavir, used alone or in association, on the increase of resorption rates seem to be dose-dependent (directly proportional). In our experiment, this association of the proposed doses did not cause important fetal alterations after its implantation in the endometrium, no deleterious effects on the quantity of fertilized eggs, nor on the embryos in the period prior to implantation.

In our study the analysis of mean fetal weight of the entire litter showed a statistically significant difference, in which experimental groups Exp 2 and Exp 3 (doses equivalent to 3 to 9 times the therapeutic dose in humans) were similar and with litter weights below those in group Exp 1 (Table 1). The association between zidovudine, lamivudine and ritonavir, used during the entire pregnancy of albino rats, was capable of causing fetal intrauterine growth restriction in experimental groups Exp 2 and Exp 3 (3 to 9 times the therapeutic dose), reflecting fetal weight gain reduction. As to placental weights, the control group, Exp 1 and Exp 2 groups proved similar and with placental weights significantly larger than those in group Exp 3 ($p = 0.045$).

A study carried out by Pontes *et al*. [16], using lamivudine at the doses of 5, 15, 45 mg/kg, showed no fetal and placental weight alterations, suggesting that alone it did not cause adverse effects on the albino rat pregnancy. In numerous human and experimental studies, zidovudine did not alter fetal and placental weights [14, 20, 21]. The experiment developed by Carvalho *et al*. [18] did not report fetal growth restriction in any of the groups using ritonavir alone during the albino rat pregnancy (doses equal to the ones used in our study).

However, Fontes *et al*. [19] studied the association

between zidovudine and ritonavir during the entire albino rat pregnancy (therapeutic dose 3 to 9 times higher) and observed that there was significant fetal growth restriction in all the groups treated with their consequent weight gain reduction.

In the last decade there has been a growing effort regarding the investigation of the proteins responsible for drug efflux through the placenta, the so-called (ABC) ATP-linked strip transporters, expressed on the syncytiotrophoblast membrane, including the P glycoprotein (gp-P), multidrug resistance-associated proteins: MRP1, MRP2, MRP3 (MRPs) and breast cancer resistance protein (BCRP) which aim is to moderate drug penetration through the placental barrier and, therefore, limit fetal exposure. Some of these transporters were originally found in cancer cells with resistance to multiple drugs; nonetheless, today the important role on the modulation of absorption, distribution and metabolism of drugs in the maternofetal interface is recognized [20, 22, 23], especially gp-P and MRP2 which have their greatest concentration on the apical border (maternal side) of the trophoblast, while MRP1 and MRP3 are located on the basal plate, in other words, in the fetal compartment [24].

In an animal experiment, BCRP expression decreases as delivery approaches. Therefore, this important transporting role can be assigned to fetal protection against drugs and xenobiotics through the placental barrier, especially in the middle of gestation; while gp-P andMRP2 expressions fall and increase respectively on the placenta as delivery approaches [25]. These mechanisms associated with the placental barrier can still be influenced by the co-administered drug-drug interaction. There can be BCRP inhibition if a drug is a BCRP substrate, while the other drug inhibits it: there would then be an increase of fetal exposure to the drug. Another important factor to be considered in this placental-drug-transportation is the regulation caused by steroid hormones, cytokines and growth factors. Progesterone may increase BCRP expression, better protecting the fetus while obstetric complications associated with cytokine elevation can increase fetal exposure. BCRP may transport a broad spectrum of substrates, among them: zidovudine and lamivudine [26, 27]. A number of BRCP inhibitors, among them, protease inhibitors such as nelfinavir and ritonavir, explaining, at least partially, placental permeability to lamivudine and the placental barrier against nelfinavir and ritonavir [28].

The discrepancies found regarding antiretroviral agents used alone or in combinations, as well as the different gp-P expressions in rats, mice and in human beings, can be explained by the different mechanisms involved in gp-P expression among the species. It seems obvious that gp-P is present in the placenta since the initial stages of gestation, changing its expression during term - which can influence the effects of xenobiotics on the fetus [29].

The association between zidovudine, lamivudine and ritonavir in our experiment did not cause any fetal malformation nor maternal or fetal deaths in the four groups studied. When zidovudine, lamivudine or ritonavir were used alone in other experiments, during the entire albino rat pregnancy, no fetal malformation or mortality was observed. However, Carvalho *et al*. [18] reported the death of four rats from a total of ten (40%) with the isolated use of ritonavir at the dose of 180 mg/kg of body weight per day. The autopsy of these rats revealed macroscopically the involvement of multiple organs (heart, lungs, liver and kidneys).

The high maternal mortality caused by the use of ritonavir alone may be attributed to the inhibition of the P-450 metabolic enzymatic system by ritonavir itself in higher doses, increasing even further its circulating levels. By adding zidovudine and lamivudine in our experiment, these mechanisms seemed to have been dampened, reducing blood levels of ritonavir and, with that, reducing maternal toxicity. Moreover, zidovudine may have activated intestinal gp-P - helping reduce serum levels of ritonavir by reducing its absorption and increasing its clearance [30, 31].

Today, many special pharmacokinetic factors which act on the maternofetal set are starting to be clarified, while information on the pharmacodynamic differences, for instance, the characteristics of the receptors and responses produced are still incomplete. Although we can still not make any direct correlation between drug toxicity in animals and human beings, the findings from our experiment suggest that the association among zidovudine, lamivudine and ritonavir cause alterations on drug pharmacokinetics. Finally, it should be stressed that with the many antiretroviral agents available, especially their use in combinations, studies must be accelerated with the aim of enhancing HIV treatment in a way to bring about maximum efficacy to prevent maternofetal transmission with minimum deleterious effects for the fetus.

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