Effects of Dilutions of *Apis mellifera* on Metabolic Abnormalities Induced by Antiretroviral Therapy in Mice

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Original Research Article

ABSTRACT

Background: Highly Active Antiretroviral Therapy (HAART) is associated with significant mortality decreased and risk in AIDS progression. However, complications due to long-standing HIV infection and treatment have become increasingly important. Complications include hepatic and nephrotoxic effects of HAART. Studies on honeybee (*Apis mellifera*) venom proved its anticancer effects, antimicrobial activity, immunomodulatory and vasoconstrictor effects. Current study evaluates the effect of dilutions of *Apis mellifera* on metabolic alterations induced in mice subjected to antiretroviral therapy (HAART).
Materials and Methods: Each experimental group comprised 10 animals: (I) animals treated with HAART diluted in 1.2 mL water gavage/day, (II) animals treated with HAART diluted in 1.2 mL water gavage/day + *Apis mellifera* diluted $1x10^{12}$ in water 1.0 mL once daily added to the drinking water (1:10 mL) available *ad libitum*, (III) animals treated with HAART diluted in 1.2 mL water gavage/day + *Apis mellifera* diluted $1x10^{60}$ in water 1.0 mL once daily added to the drinking water (1:10 mL) available *ad libitum,* (IV) untreated (control group) received 1.2 mL water by gavage/day. The experimental groups were treated for 15 days. Clinical evaluation (body weight, water intake and ration, excretion products, behavior) was performed before and after treatment and the serum cholesterol, triglycerides; hepatic enzymes (AST, ALT) and creatinine were assessed by specific methods. Results were analyzed with Graph Pad Prism using Student´s t test.
Results: Animals treated with HAART and *Apis mellifera* diluted (II and III) had higher body weight gain, lower levels of triglycerides (20%), cholesterol (20%) and creatinine (50%) when compared to animals treated with antiretroviral therapy.
Conclusion: Renal dysfunction is common in HIV-patients and studies are consistent with HAART inhibiting creatinine secretion. *Apis mellifera* diluted $1x10^{12}$ and $1x10^{60}$ showed a significant effect on creatinine levels when compared to HAART group and demonstrated possible effect on kidney injury.

Keywords: Apis mellifera; HIV/AIDS; antiretroviral; metabolic abnormalities.

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1. INTRODUCTION

Since the introduction of Highly Active Antirretroviral Therapy (HAART) has led to a dramatic decline in morbidity and mortality associated with Human Immunodeficiency Virus-1 (HIV-1) infection and Acquired Immune Deficiency Syndrome (AIDS), several complications of long-standing infection and long-term treatment have been recognized with increasing frequency. These noninfectious comorbidities include a variety of renal diseases, liver toxicity, lipodystrophy, pancreatitis, hyperlipidemia, lactic acidosis and insulin resistance [1].

In spite of the evident benefits of antiretroviral therapy and suppresses viral replication on renal function, some antiretroviral drugs can occasionally induce a reversible or irreversible renal damage.

The occurrence of various kinds of nephrotoxicity has been reported in patients treated with nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs), but the pathogenetic mechanism of renal damage remains usually unknown. Only 3 antiretroviral agents have a well-established association with direct renal toxicity sustained by several case reports and cohort studies, namely tenofovir, indinavir and atazanavir [2][3][4].

Must drugs and their metabolites are excreted through the kidneys by glomerular filtration and tubular secretion. Particularly, drug and toxin excretion usually involves the proximal tubule where there is a high rate of blood plow, and consequently this part of the nephron is at increased risk of developing drug-related injury. Moreover, proximal tubule dysfunction may be caused by a crystal-induced obstruction or by severe mitochondrial abnormalities induced by specific PIs or NRTIs. Otherwise, renal toxicity may occur in the context of an idiopathic, systemic hypersensitivity reaction. Finally, chronic metabolic complications such as diabetes mellitus and dyslipidaemia) associated with life-long antiretroviral treatment might increase the risk of vascular chronic renal disease [5-7]

Several cases of renal tubular acidosis, Fanconi syndrome and nephrogenic diabetes insipidus have been described in patients receiving didanosine, stavudine, lamivudine or abacavir. [8,9]

The traditional use of animals or their products for medicinal purposes has been documented throughout history in ancient documents such as papyri, archives, and several classical medicinal compendiums, even going back to the practices of the ancient Mesopotamian, Assyrian and Babylonian civilizations [10]. Some of the best known medicinal compendiums contain animal samples are those from Hippocrates (Greece, V-IV century BC). About 10% of the medicinal samples included in the main classical works of animals [11].

Zootherapy, or the use of animal products for the treatment of human or animal diseases, seems prevalent in certain areas of the world, particularly where traditional medicines are very important, more than allopathic medicine. This is the case for areas such as Brazil [10], Middle East [11], Turkey [12], India [13], China [14] and Korea [15]. Few studies have been undertaken on the medicinal use of animal products in Europe [16].

The study of medicinal compounds derived from animals in traditional medicines is very important, since it has been estimated that over 80% of the global population has a health system based on traditional medicine, using mainly plants and animals [17].

Honeybee (*Apis mellifera*) venom contains several enzymes, peptides and vasoactive amines [18]. Melittin is the main component in the venom of the honey bee. It was multiple effects which include antibacterial, antiviral and anti-inflammatory activities in various cell types [19].

Park et al.[20] demonstrate that honeybee venom possess a potent suppressive effect in anti-apoptotic responses of TNFα/ actinomycin D treated hepatocytes and suggest that these compounds may contribute towards a substantial therapy for the treatment of liver diseases.

Current study assess the capacity of honeybee venom diluted in experimentally induced antiretroviral toxicity in mice.

2. MATERIALS AND METHODS

2.1 Animals

Four-week old male Swiss Webster mice, weighing approximately 28-30 g, provided by the

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Central Animal Laboratory of the State University of Maringá, were used in the experiments. The Committee for Ethics in Animal Experiments of the State University of Maringá approved the experiments (Protocol number 3998020517/2017).

The animals, kept in cages with food and water *ad libitum*, were monitored daily, for 7 days, for clinical evaluation. They were kept in a vivarium of the Laboratory of Parasitology / DBS/UEM under ideal conditions: temperature 22°C ± 2°C, 70% humidity and photoperiod (light / dark cycle 12 h).

2.2 Preparation of *Apis mellifera*

The drug in the form of mother tincture and prepared from the lives Honeybees (10 unit) was mixed in 10 mL grain alcohol (P.A.) obtained from laboratory HN CRISTIANO, São Paulo, Brazil. The mother tincture contains not only the components of the bee venom but also those of the sac and glands with venom besides parts of the whole animal. As potent allergens the preparations for administration were diluted in water. The mother tincture was then diluted $1x10^{12}$ and diluted in $1x10^{60}$ of water. The method for drug preparation followed the Brazilian Homeopathic Pharmacopoeia [21]. The dilution was considered free from any toxicity.

2.3 Preparation of HAART

Protocol was based on a standard therapeutic regimen of patients from Brazil. Dose was proportional to weight of animals, as employed in humans. Treatment consisted of 167mg/kg/day of lopinavir+ritonavir(LPV/r) + zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water and tenofovir 300mg/day diluted in 1.2mL of water.

Treatment period lasted 15 days and drug was administered at 09:00 h.

2.4 Treatment Schedule

The four experimental groups with 10 animals each were distributed as follows: (I) animals treated with HAART diluted in 1.2 mL water gavage/day, (II) animals treated with HAART diluted in 1.2 mL water gavage/day + *A. mellifera* diluted $1x10^{12}$ in water 1.0 mL once a day, added to the drinking water (1:10 mL) available *ad libitum*, (III) animals treated with HAART diluted in 1.2 mL water gavage/day + *A. mellifera* diluted in $1x10^{60}$ in water 1.0 mL once a day, added to the drinking water (1:10 mL) available *ad libitum*.(IV) untreated animals (control group) received 0.2 mL water by gavage/day. The experimental groups were treated for 15 days.

2.5 Evaluation

2.5.1 Assessment of body weight

Animals were weighed on a semi-analytical balance BL320H Mars Shimadzu before the start of the treatment and at the end of the experiment. Results were given in mean of group.

2.5.2 Clinical evaluation

Qualitative parameters, such as physical appearance of the animals during the treatment (hair bristling and irritability).

2.5.3 Laboratory evaluation

Performed by plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) were evaluated by the kinetic colorimetric method; triglycerides, total cholesterol and creatinine were evaluated by enzymatic colorimetric method, both provided by GOLD ANALISA DIAGNÓSTICA LTDA.

2.6 Statistical Analysis

Group-comparing statistics were performed by Graph Pad Prism 6.0 (Graph Pad, San Diego, CA, USA) with Student´s *t* test; $p<0.05$ was statistically significant.

3. RESULTS

The antiretroviral lopinavir / ritonavir and zidovudine / lamivudine and tenofovir were used in the assays, following protocol routinely used with patients.

Experiments results agreed with those of the literature with regarding to HAART's adverse effects, or rather, lower weight gain in animals treated with HAART, higher levels of liver enzymes, cholesterol, triglycerides, and higher plasma creatinine levels.

On the other hand the results obtained in the groups treated with HAART + *Apis mellifera* showed lower alterations.

Assays revealed that the HAART group presented a weight gain lower than that of control

group. Slight weight gain in animals may be related to the already known adverse effects of the antiretroviral therapy. On the other hand, animals treated with *Apis mellifera* in diluition $1x10^{12}$ presented similar weight gain when compared to control (Table 1).

Several authors reported loss of weight loss associated with the use of HAART observed in the patients who use this therapy. Absorption deficiencies and increased energy needs are indicated as causes [22] [23].

Current results demonstrate a beneficial effect of *Apis mellifera* diluted above parameters. In the case of triglycerides levels, the $1x10^{60}$ dilution reduced levels close to those of control without HAART, whereas in the case of total cholesterol levels, the two dilutions of *Apis mellifera* showed the same reduction levels, close to control (Table 2).

Current experiments demonstrate that diluted *Apis mellifera* improved plasma creatinine levels in animals treated with HAART (Table 2).

4. DISCUSSION

Current studies have shown that melittin, a component of bee venom, has an anticancer effect on gastric cancer by stimulating the death of necrotic cells [24]. Therefore, the beneficial effect of *Apis mellifera* occurs by direct action on the cells of the digestive tube damaged by HAART.

Table 1. Weight gain (g) of Swiss mice from the experimental and control groups after 15 days of treatment

Experimental group	Initial weight(g)	Final weight(g)	Weight gain (g)	P
HAART	32.6	39.4	6.7±2.9498*	0.05
HAART + Apis mellifera$1x10^{12}$	30.6	38.8	8.2± 2.589**	0.0036
HAART + Apis mellifera $1x10^{60}$	34.2	39.8	5.6± 1.866**	0.01
Control	30.2	38.7	8.487 ± 2.495	0.001

Table 1: Weight Gain (g) of Swiss mice after 15 days. Comparison between experimental groups: treated with HAART (167mg / kg/day of lopinavir+ritonavir(LPV/r) and zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water+ tenofovir 300mg/day diluted in 1.2mL of water r; treated with HAART (167mg / kg/day of lopinavir+ritonavir(LPV/r) and zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water + tenofovir 300mg/day diluted in 1.2mL of water+ Apis mellifera$1x10^{12}$ once a day, added to drinking water (1:10 mL) available ad libitum; treated with HAART (167mg / kg/day of lopinavir+ritonavir(LPV/r) and zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water + tenofovir 300mg/day diluted in 1.2mL of water+ Apis mellifera$1x10^{60}$ once a day, added to drinking water (1:10 mL) available ad libitum;and non-treated group (control group). Results are given as mean ± SD of 10 animals

Table 2. Metabolic parameters in experimental groups

Experimental group	Lipid profile		Hepatic enzymes		Renal function
	Total Cholesterol (mg/mL)	Triglycerides (mg/mL)	AST (U/L)	ALT(U/L)	Creatinine (mg/dL)
HAART	146.0 ± 20.14*	328..2 ± 53.3*	56.97±23.78	35.82±15.02	0.702 ±0.303*
HAART + Apis mellifera$1x10^{12}$	97.15 ± 29.97*	290.15 ± 71	62.0 ± 6.02	33.1±7.78	0.450 ± 0.121*
HAART + Apis mellifera $1x10^{60}$	91.00 ± 43.14*	176.00 ± 63.3**	50.6 ±17.14	27.9±20.17**	0.330±0.1.77**
Control	94.3 ± 16.04	199 ± 30.4	43.35 ±8.36	28.10 ±15	0.302 ±0.105

Table 2: Biochemical data in the experimental groups after 15 days. Comparison between experimental groups: treated with HAART (167mg / kg/day of lopinavir+ritonavir(LPV/r) and zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water+ tenofovir 300mg/day diluted in 1.2mL of water r; treated with HAART (167mg / kg/day of lopinavir+ritonavir(LPV/r) and zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water + tenofovir 300mg/day diluted in 1.2mL of water+ Apis mellifera$1x10^{12}$ once a day, added to drinking water (1:10 mL) available ad libitum; treated with HAART (167mg / kg/day of lopinavir+ritonavir(LPV/r) and

*zidovudine/lamivudine (AZT/3TC)15mg / kg/day diluted in 1.2mL of water + tenofovir 300mg/day diluted in 1.2mL of water+ Apis melliera1x10^{60} once a day, added to drinking water (1:10 mL) available ad libitum;and non-treated group (control group). Results are given as mean ± SD of 10 animals. *p<0,05 **p<0,01 ***p<0,001*

Lopinavir, an HIV protease inhibitor, is active against HIV-1 and HIV-2. The medicinal product is only available together with low dose ritonavir formulation, to increase lopinavir concentrations and inhibit CYP3A4 metabolism [25]. According to Tavares [26], the drug is poorly tolerated at the beginning of treatment since it causes high serum triglycerides in more than 20% of patients. The most common adverse reactions are nausea, vomiting, diarrhea, tingling or numbness in the hands, feet, around the lips, headache, feeling weak or tired, or unpleasant taste in the mouth, loss of appetite, loss of appetite. Allergic reactions including mild skin rashes, bronchospasm, angioedema, and rarely anaphylaxis and allergic rhinitis, have been reported. High hepatic transaminases, exceeding five times the upper limit of normality, clinical hepatitis and jaundice occurred in patients who received ritonavir alone or combined to other antiretroviral medicinal products [27].

The literature reports several reactions caused by lamivudine: nausea, vomiting, stomach pain, diarrhea, pancreatic inflammation, headache, numbness, tingling sensation or weakness in the legs, fever, respiratory, nasal, cough and Pharyngitis, tiredness, generalized feeling of discomfort, rash (red spots and plaques from the body, itching), hair loss. Joint pains, muscle disorders including rare reports of muscle tissue rupture, anemia, neutropenia, and platelet reduction have been reported in addition to the frequent increase of liver enzymes. A case of lactic acidosis and severe hepatomegaly with steatosis (including fatal cases) have been reported with the use of lamivudine in the treatment of HIV infection [28].

In general, tenofovir is well tolerated by patients; some of the usual adverse effects are nausea (11 to 16%), vomiting (3 to 7%), abdominal pain, diarrhea (6 to 11%), flatulence, dyspepsia and anorexia (4%). It may induce mitochondrial toxicity, lactic acidosis, and elevation of transaminases, nausea and vomiting. With prolonged use it can cause changes in liver fat, lipodystrophy, headache, neuropathy, pancreatitis and anemia [1].

Clinical evaluation and weight gain demonstrated that animals treated with HAART + *Apis mellífera* 1x10^{12} presented similar results as those of control group without HAART therapy. The above suggests a beneficial / protective effect of *Apis mellífera.*

The evaluation of metabolic parameters showed a significant difference in levels of plasma triglycerides and total cholesterol in animals treated with HAART (Table 2).

Dyslipidemia is a major complication of antiretroviral treatment. HIV infection has adverse effects on lipid profiles and cardiovascular risk of HIV-positive patients. Since antiretroviral therapy increases biosynthesis and reduces hepatic clearance of serum cholesterol, the impact of antiretroviral treatment on serum lipoprotein levels should be evaluated [29].

Liver disease has emerged as the most common cause of death among HIV infected patients accounting for 14-18% of all deaths [30]. Highly active antirretroviral therapy can damage liver function. Nearly half of deaths among hospitalized HIV infected patients in the HAART era have been attributed to liver disease [31]. Liver cirrhosis is a more serious consequence with an estimate overall prevalence of 8.3% in HIV infected persons [32]. Liver disease is often reflected by biochemical abnormalities of liver function. Many authors agree that elevated serum activity of the two commonly used liver enzymes (alanine aminotransferase-ALT) and aspartate aminotransferase-AST) that are involved in breakdown of amino acids reflects liver cell injury [33].

Current experiments with animals demonstrate the effect of HAART on liver enzymes whose levels have been elevated when compared to the control group. On the other hand, in the groups of animals submitted to HAART + *Apis mellífera* 1x10^{12} a lower alteration in these parameters was observed, and ALT levels in the group HAART + *Apis mellífera* 1x10^{60} presented levels close to the control (Table 2). These experiments demonstrate the need for dilution of *Apis mellifera* and the most diluted formulation (*Apis mellífera* 1x10^{60}) showed a beneficial effect.

Melitin is the principal toxic component in the venom of the European honey bee *Apis mellifera* and is a cationic, hemolytic peptide. It

is a small linear peptide composed of 26 amino acid residues in wich the amino-terminal region is predominantly hydrophobic whereas the carboxy-terminal region is hydropholic due to the presence of a stretch of positilively charged amino acids. Melitin was reported to have inhibitory effects on hepatocellular carcinoma and inhibits tumor cell metastasis by reducing cell motility and migration via the suppression of rac-1dependent pathway [34]. Melitin can induce apoptosis of human hepatocellular carcinoma cells by activating Ca2+/calmodulin-dependent protein kinase. In presence of melitin apoptosis is significantly increased in human hepatocellular carcinoma [35].

Death of hepatocytes is a characteristics feature of chronic liver disease for various causes. Bee venom inhibited the apoptotic cell morphology and increased the cell viability in ethanol-induced hepatocyte apoptosis [36].Low concentration *Apis mellifera* venom possess a potent suppressive effect on anti-apoptotic responses of TNF-alpha/Act D-treated hepatocytes and suggest that these compounds may contribute substantial therapeutic potential for treatment of liver diseases[20].

Acute kidney and chronic kidney disease are more common in the HIV-infected population than in the general population. Renal dysfunction is common in HIV-positive patients who receive antiretroviral therapy. Glomerular and tubular diseases are often identified in HIV-infected patients. Several antiretroviral agents have been associated with the progression of kidney disease, inhibition of renal tubular transporters that mediate creatinine secretion or with impaired reabsorption of phosphate and low-molecular weight proteins. Tenofovir and atazanavir may cause acute tubular injury, tubule-interstitial nephritis or nephrolithiasis [37]. Tenofovir is associated with severe acute kidney injury in a small percentage of patients and with subclinical abnormalities in many more [38]. Some antiretroviral agents are related to kidney disease, hyperlipidemia, diabetes mellitus and hypertension which may intensify the risk of incidence of chronic kidney disease [39].

Human envenoming caused by bee stings has been reported to cause acute renal failure. Renal failure by bee venom may be related to a malfunction of renal transporters. Bee venom partly inhibits alpha–MG, Pi and Na(+) uptakes through melittin. The latter increased Ca(2+) uptake and arachidonic acid released in primary cultured rabbit renal proximal tubule cells. Bee venom (1µg/ml) decreased cell viability and increased lactate dehydrogenase activity in over 30-min treatments. However, there was no effect on cell viability at a concentration of 0.01µg/ml of bee venom [40]. Bee venom is also a complex mixture of enzymes and proteins and its diluted form suggests an improvement in renal function which could be related to the potent effect of mediators in the venom.

5. CONCLUSION

Renal dysfunction is common in HIV-patients and studies are consistent with HAART inhibiting creatinine secretion. *Apis mellifera*, diluted $1x10^{12}$ and $1x10^{60}$, showed a significant effect on creatinine levels when compared to those in HAART group, demonstrating possible effect on kidney injury.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate committee for Ethics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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