Effects of two formulations containing the IFN α2b and IFN γ on the main clinical, hematological and biochemical parameters in non-human primates from Chlorocebus aethiops sabaeus *species*

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ABSTRACT

Interferons (IFNs) are peptides with antitumoral action, widely used for the treatment of many diseases of oncologic origin. New evidences on the effect of a combination of IFN α2b and IFN γ led to the formulation, CIGB 128, containing both IFNs. A variant, CIGB 128-A, was generated through excipient optimization, to reduce the number of administrations and the undesirable effects of the product. In this study, the effects of both products, CIGB 128 and CIGB 128-A, were evaluated attending to the behavior of clinical, hematological and biochemical parameters in *Chlorocebus aethiops* non-human primates. There were no clinical alterations on the body weight, major hematological parameters and the transaminases levels. The creatinine levels showed a slight decrease, related to the treatment with the formulations. These results provide new safety data on the effect of CIGB 128 and CIGB 128-A, supported by the unaltered main clinical, hematological and biochemical parameters in *C. aethiops*. They further indicate their potentially safe profile to test both formulations in humans.

Keywords: interferons, hemalotogical parameters, biochemical parameters, non-human primates, *Chlorocebus aethiops*, CIGB 128, CIGB 128-A

RESUMEN

Efecto de dos formulaciones con mezclas de IFN α2b e IFN γ sobre los parámetros clínicos, hematológicos y hemoquímicos en primates no humanos *Chlorocebus aethiops sabaeus***.** Los interferones (IFNs) son péptidos de acción antitumoral ampliamente utilizados en el tratamiento de muchas enfermedades de origen oncológico. A partir de nuevas evidencias sobre el efecto sinérgico de la combinación del IFN α2b y el IFN γ, se desarrolló una nueva formulación denominada CIGB 128. Una variante de esta, el CIGB 128 A, contiene a ambos interferones formulados con excipientes optimizados, lo permite disminuir el número de administraciones y los efectos indeseables. En este estudio, se evaluaron los efectos de ambos productos, CIGB 128 y CIGB 128-A, atendiendo a los parámetros clínicos, hematológicos y hemoquímicos, tras su administración en monos de la especie *Chlorocebus aethiops*. No se detectaron afectaciones en los primates no humanos durante la evaluación clínica, ni en el peso corporal, ni en varios de los principales hematológicos y las transaminasas. Los niveles de creatinina mostraron una ligera disminución, asociada a los tratamientos con estas formulaciones. Estos elementos avalan la seguridad de la administración del CIGB 128 y CIGB 128-A en *C. aethiops* y evidencian su posible perfil de seguridad en seres humanos.

Palabras clave: interferones, parámetros hematológicos, parámetros hemoquímicos, primates no humanos, *Chlorocebus aethiops*, CIGB 128, CIGB-128-A

Introduction

Interferons are natural proteins displaying unspecific antiviral action on homologous cells, which have been isolated, obtained in recombinant form and used as antitumor agents in the therapeutic setting, as a powerful tool to aid the natural defense system of the organism against certain tumor types and infections [1]. They have been classified attending to their cell origin, genetic phylogeny and protein properties in two major types: type I (IFN α and ß) and type II (IFN γ) [2, 3]. Type I IFNs bear a higher antiproliferative and antiviral effects, while type II IFNs develops a significant regulatory activity [4, 5]. These molecules have demonstrated a direct action on a myriad of tumor cell lines mediated by their cytostatic effect [6]. Moreover, the increased activity of natural killer cells and the antibody-dependent cell cytotoxicity (ADCC) processes enhanced by IFNs have been considered as the major indirect effector involved in cancer therapy.

IFNs are species-specific proteins. Noteworthy, human IFNs have no effect on murine cells [7], while still active to a certain extent in some of the monkey species used for their preclinical validation prior to its clinical application [8, 9]. This is significant since non-human primates (NHPs) have become an essential tool in biomedical research, with the Old World monkey species as the most phylogenetically related to human beings. The African green monkey (*Chlorocebus aethiops sabaeus*) is one of the most widely used species for animal experimentation, which has been recently declared as relevant for pharmacological studies before conducting clinical trials [10].

More specifically, rhesus monkeys (*Macaca mulatta*) biologically respond to IFN β, being a useful preclinical model for studying the pharmacokinetics and pharmacodynamics of IFNs *in vivo* [11]. Green monkeys are also responsive to the action of human

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IFNs [12]. Several *in vitro* studies further supports such considerations, with Vero cells from green monkey kidney which do not secrete IFN in response to viral infections [13] being responsive through its IFN receptor to the addition of human IFN to the culture medium, this antiviral response been enhanced by adding a synergic mixture of IFNs α 2b and IFN γ [14].

In spite of such experimental evidences on the synergistic effect of IFNs combinations *in vitro*, as far as we know, there are no previous references on the combined use of recombinant human IFN γ and IFN α 2b inoculated by the intratumoral route for the treatment of gliomas. This combination shows promising results for the synergistic antiproliferative effects on various tumor cell lines [15], but it has to be tested for improving its pharmacodynamic, pharmacokinetic and safety properties as a single formulation, in order to test it in humans. A formulation was recently developed at the Center for Genetic Engineering and Biotechnology (CIGB; Havana, Cuba), named CIGB 128, containing both types of human IFNs, together with a formulation variant denominated CIGB 128-A which excipients were optimized to reduce the number and frequency of administrations and also the occurrence of its adverse effects.

Therefore, this work was aimed to comparatively evaluate the effect of the CIGB 128 and CIGB 128-A on the clinical, hematological and biochemical parameters of NHPs of the *C. aethiops* species. According to the literature, this is the first trial evaluating such an IFN combination in this species.

Materials and methods

Animal study

Seven male *C. aethiops* monkeys were used, apparently healthy, 3 to 10 years-old (young adults). They were taken from the colony of non-human primates (NHPs) at CIGB, and verified as tuberculosis- and herpes virus B-free by serological testing.

Study design

Due to the small number of animals included in the study, they were subjected to simple randomization in two groups: I) four NHPs (1, 4, 5 and 7); and II) three NHPs (2, 3 and 6) (Figure 1). Both formulations, CIGB-128 and CIGB-128-A were cross-tested, considering their half lifetime. The study was made in two phases, 192 h each, with an interval of two weeks in between as required for the clearance from plasma to avoid the effects of these products [16, 17]. At start (time 0), the moment of the first administration of the formulations, animals in group I were administered with CIGB-128, and immediately after the intermediate two-week period (the start of the second phase), with CIGB-128-A. Animals in group II were treated with both formulations in reverse order (Figure 1).

The CIGB-128 and CIGB-128-A formulations were prepared by mixing synergic (equivalent therapeutic) amounts of IFN α2b and IFN γ, 3.5 mIU each in a final volume of 1 mL [18]. They were immediately administered by intramuscular route in the tricep regions. All the procedures were performed by well-trained personnel skilled in handling NHP, and complying with ethics institutional guidelines.
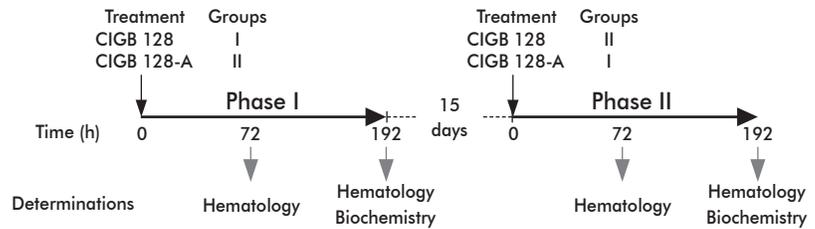
Figure 1. Treatment schedule for determining the effect of two formulations, CIGB 128 or CIGB 128-A, containing the IFN α2b and IFN γ, administered to *Chlorocebus aethiops sabaeus* non-human primates (NHPs). CIGB 128-A was obtained from CIGB 128 by optimizing formulation excipients. During each phase, all the NHPs were also clinically inspected at 24, 48, 72, 96 and 192 h, in addition to hematology and biochemistry evaluations.

Animal housing and management

The assay was performed within the NHPs experimentation area, at the stabled animals in the Animal Facility of the CIGB, where the monkeys were kept under conventional housing conditions. Animals were always exposed to 22-29 °C during the study and housed individually in single stainless steel cages (Inpud, Cuba). Under these conditions, animals can view, hear and smell other NHPs of the same species.

NHPs were fed with a commercial concentrate, formula CMO 1600 purchased from the National Center for the Production of Laboratory Animals (Cenpalab, Cuba), provided at 350 g of the concentrate per animal in two daily rations and water *ad libitum*. Additionally, seasonal fruits were provided once a day.

Before each treatment, NHPs were sedated with a ketamine chlorhydrate injection at a dosage of 10 mg/kg of body weight.

Observation and clinical inspection of animals

Animals were clinically inspected daily to evaluate the occurrence of any change in mobility or behavior, evidences of pain and their patterns of food and water consumption. For that purpose, a clinical inspection was conducted at the start and 24, 48, 72, 96 and 192 h after the administration of the each formulation on the respective phase of the study. The clinical status of the skin, fur, mucosal tissues (conjunctiva, nasal, oral, auditory, genital and rectal), lymph node, genital-urine system, and the gastrointestinal, respiratory, cardiovascular and nervous systems. These operations were conducted according to the Program for the Use and Handling of Laboratory Animals for Experimental Purposes and for the Control of Biotechnological Products at the CIGB, Cuba [19].

Body weight and rectal temperature measurements

Body weight and rectal temperature were also determined at the same timepoints as the clinical inspection. For that purposes, a previously calibrated scale and mercury bulb thermometers (Sartorius, Germany) were used, respectively.

Hematological and biochemical analyses

Hematological parameters were evaluated at 72 and 192 h of each phase, and the biochemical determinations only after 192 h, for each phase (Figure 1).

Four milliliters of blood were extracted from each animal with 10-mL syringes and 21 G × ½ gauge

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needles. Immediately after the extraction, blood was transferred to test tubes containing EDTA as anticoagulant. Hematologic parameter determinations included the analysis of total leukocyte count (WBC), total erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit percentage (HCT), platelet total count (PLT). Blood samples were evaluated in a hematology analyzer Nihon Kohden (Celltac model MEK6450 J, Japan). A differential leukocyte count (measured as percentage of WBC) including neutrophils (NEUTRO%), lymphocytes (LYMPHO%), monocytes (MONO%), eosynophils (EO%) and basophyls (BASO%) was performed by stained peripheral blood films with Giemsa reactive and cells were counted using an optical microscope equipped with immersion lens (VistaVision, MO 000004, Zeiss, Germany).

Blood samples were kept at room temperature complete retraction of the clot, further centrifuged at 10 000 *g* for 15 min, and sera was collected and stored at –20 ºC until use.

Serum biochemical parameter analysis included the evaluation of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine (CREA). These measurements were performed with commercial reagents from SPINREACT (Barcelona, Spain) following the method recommended by the manufacturers and using a spectrophotometer Ultrospec 2000 (Pharmacia Biotech, Cambridge, England).

Tests were performed in the Clinical Laboratory of the Animal Facility Department, CIGB, and historical data on the physiological values of healthy *C. aethiops* NHPs kept at the CIGB were used as reference values.

*R*esults

Clinical inspection and observation

Throughout the study, all the NHPs behave as normally healthy animals, without any behavioral abnormality. The clinical inspection of the organs and systems of organs (mucosa, lymphatic ganglia, skin color and texture, respiratory, digestive and nervous system, and somatomotor activity) reflected a normal, unaltered physiological state.

Body weight and rectal temperature

In spite of the daily chemical sedation and the blood extractions performed, the animals kept a stable body weight throughout the study, with few transient fluctuations and an overall higher body weight at the end of the study, as compared to recorded data at the start (Figure 2A and B).

Similarly, body temperature was observed within the physiological range reported for the species (37 to 40 °C), with just slightly increased values in two monkeys, NHP 5 at 24 h following the administration of CIGB 128 and NHP 6 at 48 h after receiving CIGB 128-A.

Analysis of hematological parameters

Total WBC were found decreased in NHP 7 of group I after 192 h after the administration of CIGB 128 (Table 1). Regarding the different WBC populations, NEUTRO% were high in NHP 2 and NHP 6, 72 h and 192 h after administering the CIGB 128 formulation, also high in NHP 2 after 192 h of administering CIGB
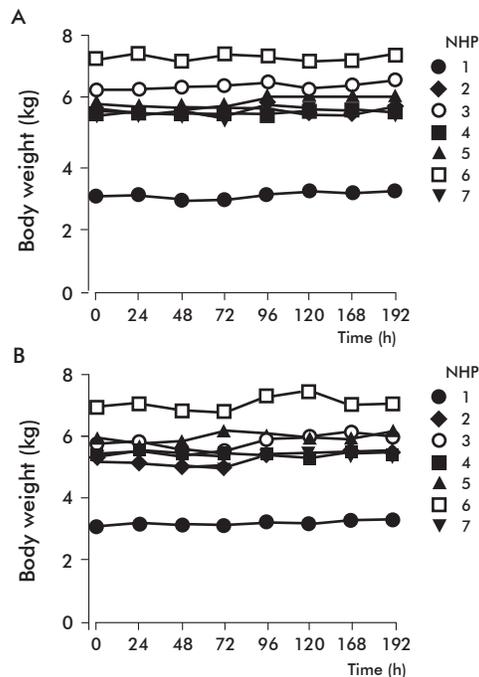
Figure 2. Performance of body weight (kg) of *Chlorocebus aethiops sabaeus* non-human primates (NHPs) treated with two formulations containing a combination of recombinant human IFN α2b and IFN g. A) CIGB 128 formulation. B) CIGB 128-A formulation.

128-A. On the contrary, LYMPHO% decreased in both NHP 2 and 6, in response to CIGB 128 after 72 h, remaining low in NHP 2 after 192 h. NEUTRO% decreased in NHP 1 and NHP 7, 72 h after treatment with CIGB 128, but high values of LYMPHO% were found in both NHPs 1 and 7.

In the case of BASO%, their levels increased in PNHs 3 and 7, 72 h after receiving CIGB 128, and in NHPs 3, 5 and 6 at 192 h (Table 1). The same effect was seen only in NPHs 3, 4 and 7 at 72 h of administering CIGB 128-A.

MONO% and BASO% remained within the physiological limits described for the species and sex (Table 1).

RBC counts decreased in response to CIGB 128 in NHP 2 at 72 h and in NHPs 1, 2, 4 and 7 at 192 h, this parameter found similarly low for CIGB 128-A in NHP 2 at this last time point.

HGB also decreased after the administration of CIGB 128 in NHP 2 after 72 h and in NHPs 1, 2, 3, 4, 5 and 7 at 192 h. The same effect was shown in NHPs 2 and 3 for CIGB 128-A at 192 h. HCT decreased in response to CIGB 128 in NHPs 1 and 7 at 192 h, but remained under physiological values for all the animals treated with CIGB 128-A. Platelets values were only detected low in NHP 7 after 192 h for the CIGB 128 treatment.

Analysis of biochemical parameters

Transaminases levels (ALT and AST) were kept within the physiological range described for the species, in all the NHPs and timepoints (Table 2). Creatinine levels decreased as compared to reference values in

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Table 1. Hematological parameters of *Chlorocebus aethiops sabaeus* non-human primates (NHP) treated with the CIGB 128 or CIGB 128-A formulations containing a combination of recombinant human IFN α2b and IFN γ

NHP	WBC (10³/µL)				RBC (10⁶/µL)				HGB (g/dL)				HCT (%)				PLT (10³/µL)			
	CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A	
	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h
1	4.4	5.2	6.2	4.2	5.20	4.24	5.33	5.58	13.5	10.7	13.7	13.4	40.5	33.6	41.6	41.5	472	527	407	293
2	4.7	4.9	3.5	3.9	4.78	4.63	5.69	4.76	12.6	12.2	15.4	12.2	38.7	38.5	45.0	38.0	128	318	177	210
3	5.3	5.9	8.0	5.4	5.69	5.22	5.88	5.07	14.0	12.9	14.7	12.3	43.4	39.9	44.3	39.1	283	296	278	250
4	3.2	4.3	3.4	5.1	5.72	4.64	5.59	5.26	14.8	11.9	14.3	13.6	44.7	36.7	44.0	41.3	293	219	321	338
5	4.1	4.3	4.3	4.5	5.79	5.06	5.95	5.51	14.9	12.6	14.9	13.8	45.1	39.5	46.6	42.9	221	226	227	269
6	3.7	5.6	5.0	3.8	6.77	6.22	6.51	6.13	17.0	15.7	16.9	15.3	52.3	47.9	50.4	47.4	299	314	149	242
7	3.0	2.8	5.0	5.1	5.97	4.49	5.79	5.56	15.1	11.5	14.2	13.5	46.0	35.0	43.9	42.1	186	106	120	286
Range[a]	2.91-12.79				5.02-6.9				13.01-17.03				36.42-53.75				116-530			

NHP	NEUTRO (%)				LYMPHO (%)				MONO (%)				EO (%)				BASO (%)			
	CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A		CIGB 128		CIGB 128-A	
	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h	72 h	192 h
1	0.12	0.20	0.29	0.22	0.85	0.79	0.70	0.72	0	0	0	0	0	0	0	0	0	0	0	0
2	0.51	0.45	0.19	0.16	0.49	0.54	0.74	0.83	0	0	0.01	0.01	0.02	0	0	0.01	0	0	0.01	0
3	0.41	0.31	0.25	0.29	0.58	0.65	0.71	0.71	0	0	0.01	0	0.03	0.03	0.05	0.01	0	0	0.01	0
4	0.16	0.43	0.21	0.35	0.82	0.80	0.78	0.63	0	0	0	0	0	0.01	0.05	0	0	0	0	0
5	0.25	0.20	0.17	0.23	0.71	0.80	0.83	0.76	0.01	0	0	0	0.01	0.04	0.02	0	0.01	0	0	0
6	0.44	0.51	0.38	0.44	0.53	0.46	0.56	0.55	0	0	0	0	0	0.04	0.01	0.01	0	0	0.01	0
7	0.14	0.20	ND	0.37	0.85	0.75	0.60	0.62	0	0.01	0	0	0.03	0.01	0.04	0	0.01	0	0	0
Range[a]	0.15-0.43				0.54-0.84				0-0.03				0-0.02				0-0.01			

[a] Physiological range as reported for the species. Values outside the physiological range are highlighted in gray boxes.
WBC: total leukocyte count. RBC: red blood cells. HGB: hemogloblin. HCT: hematocrit. PLT: platelets. NEUTRO: neutrophils. LYMPHO: lymphocytes. MONO: monocytes. EO: eosinophils. BASO: basophils. ND: not determined.

PNHs 2, 4, 5, 6 and 7 at 192 h after the administration of the CIGB 128, as well as in PNHs 3 and 4 in the group receiving the CIGB 128-A at 192 h (Table 2).

Discussion

It is relevant to notice that after the subsequent administration of CIGB 128 and CIGB 128-A, neither behavioral changes were manifested by the animals, nor evidences of any abnormal process were found in any organ or organ system, but just those corresponding to the normal physiology as evidenced the experimental observation and the clinical inspection. Whereas, reports by Felger *et al.* [20] detected signs of increased anxiety, decreased exploratory behavior, depression, slow movements and fatigue in rhesus monkeys after the administration of 20 mIU of IFN α/m² of body surface. Discrepancies found between both studies could be associated to application of IFN α alone or in combination, the NHP species tested or the treatment dosage.

The administration of IFN α2ß in humans commonly causes the loss of body weight [21]. Here, the body weight remained stable regardless the administered product, being even higher at the end of each evaluation phase as compared to the starting values for all the animals. These results demonstrated that none of the formulations at the dosages tested affected this parameter, indicating the proper handling and the management of the animals during the study, to avoid any stressing situation that could influence on their physiological response to treatment beyond the direct action of the tested products as reported by Beilharz *et al.* [22].

In humans, body temperature changes have been recorded when administering α IFNs [23-25]. Nevertheless, such changes were not found in previous studies in *M. mulatta* NPHs inoculated with the same molecule. On the contrary, there were reports on temperature increases in chimpanzees, at 4 h following treatment with IFNs [26]. Considering that temperature fluctuations were only detected in two animals and at 24 h of receiving either CIGB 128 or CIGB 128-A formulations, that period higher than previously reported, those temperature changes were regarded as unrelated to the substance under assay. Moreover, the body temperature increases were mild and no pathological alterations were found, indicating that the changes could be an effect of the ketamine chlorhydrate sedative, as previously reported [27, 28].

Regarding leukocytes, these cells are immune cells involved in fighting infections and their populations are indicative of the severity of the ongoing infections when present [29, 30]. A statistically non-significant decrease has been reported in rhesus monkeys following the administration of IFN α2ß [26], in spite of similar reductions been found among the most frequent reactions in humans [24, 25, 31-33]. Just a slight decrease in WBC counts was found in a single animal after receiving CIGB 128, which was considered

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Table 2. Biochemical parameters of *Chlorocebus aethiops sabaeus* non-human primates (NHP) treated with the CIGB 128 or CIGB 128-A formulations containing a combination of recombinant human IFN α2b and IFN γ, 192 h after treatment

NHP	Biochemical parameters					
	ALAT (IU/L)		ASAT (IU/L)		CREA (mg/dL)	
	CIGB 128	CIGB 128-A	CIGB 128	CIGB 128-A	CIGB 128	CIGB 128-A
1	5.28	1.77	12.30	17.54	0.86	0.63
2	4.11	2.35	11.74	16.40	0.57	0.86
3	1.76	2.35	8.80	20.50	0.63	0.57
4	4.11	2.94	9.37	13.46	0.40	0.51
5	3.52	1.77	15.23	11.14	0.40	0.63
6	2.35	2.34	7.63	19.33	0.40	0.63
7	2.35	1.18	11.72	7.62	0.51	0.91
Range[a]	1.00-29.41		6.74-52.96		0.62-1.04	

[a] Physiological range as reported for the species. Values outside the physiological range are highlighted in gray boxes. ALAT: alanine aminotransferase. ASAT: Aspartate aminotransferase. CREA: creatinine.

not associated to the formulations administered, due to its low representativeness and the absence of any other pathological manifestation.

Similarly, the raise in neutrophils' populations is considered indicative of bacterial infections [34]. Nevertheless, only slight increases in the values were detected after the administration of CIGB 128, which neither coincided with any simultaneous increase in WBC counts nor any clinical pathological manifestations in the animals. Thus, the values observed in this parameter were considered unrelated to the evaluated products.

Neutropenia has been also detected in humans, produced after the administration of pegylated IFN α2b [35]. Curiously, this manifestation was found in just two animals at 72 h after the administration of CIGB 128. It was mild and short-lasting, since had disappeared at 192 h. Hence, this transient decrease in neutropenia was regarded as unrelated to any of the formulations studied and could have been associated to the use of ketamin chlorhydrate as sedative [36].

Since LYMPHO% were also found elevated at 72 h in two animals but showing physiological white blood cell counts, in spite of literature reports for this after the administration of IFNs, our findings could be associated to the sedative substance. Moreover, decreased WBC counts coincided with an increase in the neutrophils fraction, leading to a proportional decrease in lymphocyte counts without affecting WBC.

Additionally, the raise in EO% was considered a consequence of a mild allergic process or being generated by the IFN component present in both formulations, all these due to the lack of any clinical signs of parasitosis and based on previous reports relating eosinophilic processes to IFN administration [37, 38].

Low values for components of the red series (RBC, HGB and HCT) have been also reported in humans following the combined administration of IFN α2b and IFN γ [25], but after the fourth week of treatment [39]. Therefore, the low values observed in the animals at 72 and 192 h after treatment with either formulation were regarded as derived from the repeated administration of the ketamine chlorhydrate sedative and unrelated to any of the substances tested. This conclusion was further supported by the slight decrease observed, when present, and also to reports of such depressive effects of this sedative on the evaluated parameters [40].

In humans, PLT counts could decrease in response to treatment with IFN [25], similar to results obtained in animals of the *M. mulatta* species treated receiving IFN β-1 [41]. Even when such a reduction was found in one animal, it was slight and no clinical signs of coagulation or any other platelet disorders were found, suggesting that the observed variations could have no clinical implications.

The raise of transaminases levels (ALT and AST) could be another possible effect of the administration of IFNs in humans [25], even when such changes have not been reported in NHPs [20]. High ALT and AST levels are indicative of hepatic damage [42-44]. Similarly, the levels of these transaminases were found normal in our study, indicating that none of both formulations, CIGB 128 or CIGB 128-A, did not produce hepatic damage.

The lower creatinine levels, found in animals bearing a good nutritional state and showing no sign of renal pathological alterations, indicated that such effects were caused by neither an inappropriate diet nor any significant renal alteration [45, 46]. The fact that the aforementioned reductions in creatinine values followed the administration of either IFN formulations could indicate that the therapeutic administration of CIGB 128 and CIGB 128-A may reduce creatinine levels.

Conclusions

The administration of both formulations CIGB 128 and CIGB 128-A, containing IFN α2b and IFN γ, were demonstrated to keep unaltered the clinical parameters tested in *C. aethiops*. In spite of the increase in neutrophils and lymphocyte levels for CIGB 128, and the decreased creatinine levels for both formulations, only slight changes were recorded. Therefore, both formulations can be regarded as safe, further supporting their testing in humans.

Acknowledgements

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