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Research Article

Jusvinza, a Peptide with Activity against Hyperinflammation Process Induced by SARS-CoV-2, is Safe in Macaca fascicularis Monkeys

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Abstract

Jusvinza is a peptide, derived from the human heat shock protein 60, which could plays a key role in the immune response regulation in COVID-19 patients. Thus, the main subject of this study was assessing safety of Jusvinza in *Macaca fascicularis* monkeys as part of preclinical trials. As general methodology, Jusvinza was daily administered intravenously in monkeys for 28 days, in a similar scheme than those that could be applied in COVID-19 patients. Monkeys were separated in four groups: Placebo, Jusvinza at low dose (0.35 mg/kg), Jusvinza at high dose (0.70 mg/kg) and Reversion (0.70 mg/kg) to be subjected to a clinical and behavioral, hematological and serum biochemical evaluation as well as a histopathology assessment in all tissues and organs. As main results all monkeys survived and clinical alterations were not detected in response to Jusvinza application. The monkey corporal weight and rectal temperature did not suffer variations and hematological and serum biochemical parameters did not present alterations associated with Jusvinza application either. On the other hand, histopathological study confirmed hyperplasia of lymphoid follicles of the spleen in animal administered only with the high dose of Jusvinza. As main conclusion, Jusvinza does not cause toxic effects and damages in any studied organs of *Macaca fascicularis* monkeys indicating that it is a safe biotechnology product in animals and also most likely in COVID-19 patients.

***Keywords**: COVID-19; Jusvinza; Macaca fascicularis Monkeys; Preclinical Trial; Toxicology*

Introduction

The COVID-19 is an infectious illness caused by SARS-CoV-2 that generates severe acute respiratory syndrome in humans [1]. It was first detected in Wuhan (Chine) in December 2019 [2] and declared as pandemic by the World Health Organization on March 11, 2020 [3].

This virus produces flu-like symptoms, including fever, cough, dyspnea, myalgia, and asthenia [4]. COVID-19 can also be characterized by pneumonia, sepsis, and septic shock that can lead to patient death [5]. Until September 2022, more than 6 million deaths have been recorded as consequence of COVID-19 [6].

However, COVID-19 does not have a specific treatment. The main therapeutic effects of applied treatments consist of relieving symptoms and maintaining vital functions [4]. Likewise, the scientific literature describes a group of patients characterized by a state of hyperinflammation evolving to severe states of disease. This complex process (hyperinflammation) is known as a cytokine release syndrome, also called cytokine storm, which is a life-threatening systemic inflammatory syndrome involving elevated levels of circulating cytokines (such as: IL-2, IL-17, TNFα and IL-6) and an immune-cell hyperactivation [7]. As consequence, hyperinflammation can lead to a fatal outcome in COVID-19 patients [8]. In this scenario, the hyperinflammation treatment is hardly recommended, with the aim of reducing mortality.

Some approved drugs for the autoimmune disease treatment have been also applied in COVID-19 patients, such as mononuclear antibodies against IL-1 (Anakinra), IL-6 (Tocilizumab) and Janus kinase inhibitors [8]. Though, these treatments may eliminate hyperinflammation, could cause immunosuppression [9], which is not recommended in viral infections, because it can stimulate a potential viral outbreak [10].

Therefore, some other alternative drugs have been also intensively assessed. For instance, the Center for Genetic Engineering and Biotechnology (CIGB) of Havana, Cuba, developed Jusvinza a product that uses the CIGB-814 peptide as active pharmaceutical ingredient. Summarizing, it is a peptide derived from the human heat shock protein 60 that may induce regulatory effects associated with inhibition of inflammation in several experimental inflammatory models and in patients with rheumatoid arthritis (RA) [11].

Concerning this, Jusvinza received Authorization for Emergency Use by the Cuban Regulatory Authority for the treatment of severe and critically ill COVID-19 patients in Cuba [10]. Reason why; a repeated dose toxicity study in the Non-Human Primates (NHP) *Macaca fascicularis* monkeys was conducted previously as part of the preclinical safety program, where two doses of Jusvinza were administered and its effects on behavior, body weight, temperature, hematological, serum biochemical parameters and histopathology of all tissues and organs were assessed. Results of this comprehensive study are described in this paper.

Materials and Methods

Jusvinza

It is a lyophilized formulation containing 2.5 mg of peptide and 20 mg of sucrose dissolved in a HAc-NaOH buffer solution. This product is formulated in airtight vials and supplied together with a 1 mL-vial of water for injection.

Animals

Twenty-two adult and healthy NHP (11 males and 11 females) of *Macaca fascicularis* monkeys were used. Monkeys were captive born in Cuba and the average body weight measured at the beginning of study was 4.96 kg (males) and 3.34 kg (females). The age of monkeys ranged from 3 to 5 years old at the beginning of the study; and hematological and serum biochemical parameter values were within the physiological range for the species and sex.

Monkey handling and husbandry

Monkeys were exposed to 22 - 29°C throughout the whole study and housed individually in stainless steel cages (90 cm × 60 cm × 60 cm). Under these conditions, animals saw, heard and smelled other NHP of the same species. Monkeys were maintained on a 12/12h light/dark cycle under an environmental enrichment regime with toys and foraging enrichment during 2h per day. Monkeys were fed with fresh fruits and a commercial diet (granulated formula CMQ 1600 ALYco certified by the National Center for Laboratory Animal Breeding

(CENPALAB), Havana, Cuba, containing 25% protein, 3.5% crude fat and 3.8% crude fiber) twice daily at a rate of 150 - 300g per monkey according to respective age and body weight. Water was always provided *ad libitum* and monkeys were cared following guidelines of the American Association for the Accreditation of Laboratory Animal Care (AAALAC). The whole study was conducted under the approval of the CIGB Animal Care and Use Committee, protocol number: CICUAL/CIGB/20052.

Monkey clinical evaluation

Firstly, monkeys underwent a pre-acceptance process to be included in the study, where a skin test for tuberculin was performed. In addition, monkeys were treated with Ivermectin (200 µg/kg subcutaneously, in the interscapular region of the back). During this process, monkeys were subjected to daily clinical observations. Besides, a complete clinical examination was performed, including examination of skin, hair, mucous membranes (conjunctiva, nasal, oral, auditory, genital and rectal), lymphatic system, genitourinary system, digestive system, respiratory system, cardiovascular system and nervous system. The body temperature and weight of monkeys were measured and monkeys were also subjected to bacteriological and parasitological studies taking samples of rectal, vaginal, preputial, auricular and oral mucosal exudates and fecal samples. Hematological and serum biochemical parameters were also analyzed. As inclusion criterion, only monkeys without behavioral alteration (stereotyped movements, aggression, self-harm, apathetic/depressed behavior, over-grooming, drinking urine, eating faeces, etc.) and clinically healthy were included in the study. During the study, monkeys were subjected to daily clinical observations, similar to those performed in the pre-acceptance process. The body weight was measured with a Sartorius balance (EB Model, Sartorius, Goettingen, Germany) and temperature was measured with mercury bulb thermometer. To perform both measurements, monkeys were sedated with an intramuscular injection of ketamine hydrochloride (ketamine - 50, Liorad, 10 mL, 50 mg/mL) into the biceps femoris muscle at a dose of 10 mg/kg body weight. This procedure was performed according to the Program for the Use and Handling of Laboratory Animals for Experimentation and Control of Biotechnological Products CIGB, Cuba.

Study design

Monkeys were randomly distributed in four groups, Placebo, Low-Dose, High-Dose and Reversion. The Placebo, Low-Dose and High-Dose groups consisted of six monkeys each (three males and three females), while Reversion group consisted of only four monkeys (two males and two females). The Low-Dose group received 0.35 mg/kg and the resting two other groups received 0.70 mg/kg (High-Dose and Reversion) (Table 1). In the Reversion group, reversion of possible alterations or lesions detected in animals one month after the end of the Jusvinza application was analyzed. Jusvinza doses were chosen based on results of previous pharmacology and toxicological studies. Calculation of the estimated animal dose (EAD) was performed taking into account animal species, giving a value of 0.1770 mg/kg and setting a maximum volume in 24h. The lower dose level was determined following a mathematical criterion of x/2, leaving 2 and 4 times the NHP corresponding to 0.35 and 0.70 mg/kg, which according to the average body adjusts to 2 and 4 mg of peptide. Monkeys received Jusvinza intravenously daily for 28 days, to be immediately euthanized, with the exception of Reversion group. Monkeys of this group were euthanized 28 days later. During the study, food and water avidity and body weight were studied. Clinical observations, behavior and pathological findings in all organs were also analyzed by macro and microscopic observations. A study of different serum hematological and biochemical parameters was also carried out. As monkeys of High-Dose and Reversion groups received the same Jusvinza dosage, results of the analysis of rectal temperature, hematological and serum biochemical parameters at 28 days of both groups were integrated.

Justification of jusvinza administration route

The intravenous route was chosen, because it is the same route that will be used in human clinical trials. In detail, monkeys were intravenously dosed in the saphenous vein, alternating between the left and right saphenous vein.

Treatment/Group	Dose (mg/kg)	Animals by gender
Placebo	-	3 males 3 females
Jusvinza Low-Dose	0.35	3 males 3 females
Jusvinza High-Dose	0.70	3 males 3 females
Jusvinza Reversion	0.70	2 males 2 females

Table 1: *Study design.*

Blood sample collection from monkeys

Blood samples were collected prior to the first Jusvinza administration and at weeks 4 and 8 of the study. After an overnight fasting period (14 - 16h), monkeys were sedated with an intramuscular injection of ketamine hydrochloride (ketamine-50, Liorad, 10 mL, 50 mg/mL) into the biceps femoris muscle at a dose of 10 mg/kg body weight. Four milliliters of blood were collected from the femoral veins using 21 G × 1/2 gauge needles and 10 mL syringe. Samples were then distributed into 1 mL and 3 mL-aliquots, respectively. The 1 mL-aliquot was transferred to tubes containing EDTA as anticoagulant for the hematological parameter determination. On the other hand, 3 mL-aliquots were stored in plastic tubes without anticoagulant for the biochemical parameter determination. Aliquots were allowed to clot at room temperature for 30 - 60 minutes and sera were separated by centrifugation at 1600 xg for 15 minutes in a centrifuge 5810 (Eppendorf, Hamburg, Germany). Individual serum samples were stored in polypropylene tubes at -20°C until the moment of the analysis.

Hematological and serum biochemical parameter determination

The hematological parameter study involved the total leukocyte (WBC) and differential leukocyte count (measured as percentage of white blood cells) that included lymphocytes (LYMPHO%), neutrophils (NEUTRO%), monocytes (MONO%), eosinophils (EO%) and basophils (BASO%). Total erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit percentage (HCT), mean corpuscular volume (MCV), mean hemoglobin concentration (MCH), mean corpuscular hemoglobin concentration (MCHC) and total platelet count (PLT) were also determined. These determination were done in a Nihon Kohden hematology analyzer (Celltac model MEK6450J; Nishiochiai, Shinjuku - ku, Japan). The differential leukocyte count (measured as percentage of white blood cells) including LYMPHO%, NEUTRO%, MONO%, EO%, and BASO% was performed by staining peripheral blood slides with Giemsa reagent, and cells were counted using an optical microscope equipped with an immersion lens (VistaVision, MO 000004, Zeiss, Germany). Serum biochemical parameters were determined in a HESKA DRI-CHEM 7000 automated analyzer (FUJI FILM, FUJI DRI-CHEM 7000 Iv, MINATO-KU. TOKYO, JAPAN) and the determinations included evaluation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltranspeptidase (GGT), alkaline phosphatase (ALP) creatinine (CREA), uric acid (UA), urea (UR), total protein (TP), albumin (ALB), A/G ratio, glucose (GLU), triglycerides (TG), cholesterol (CHOL), direct bilirubin (BIL-D), total bilirubin (BIL-T), calcium (CA), phosphorus (PHOS), cholinesterase (CHE) and pancreatic amylase (P-AMY). Abbreviations and units are described in table 2.

Euthanasia

Monkeys were euthanized by intravenous administration of 200 mg/kg sodium thiopental (Sodium Thiopental-500, AICA, 500 mg) after sedation with ketamine hydrochloride (ketamine-50, Liorad, 10 mL, 50 mg/mL) following recommendations of the Program for the Use and Handling of Laboratory Animals for Experimentation and Control of Biotechnological Products CIGB, Cuba.

Parameters	Abbreviation	Unit
White Blood Cells	WBC	$10^3/\mu L$
Red Blood Cells	RBC	$10^6/\mu L$
Hemoglobin	HGB	g/dL
Hematocrit	HCT	%
Mean Corpuscular Volume	MCV	fL
Mean Corpuscular Hemoglobin	MCH	Pg
Mean Corpuscular Hemoglobin Concentration	MCHC	g/dL
Platelet Count	PLT	$10^3/\mu L$
Neutrophils percentage	NEUTRO%	%
Lymphocytes percentage	LYMPHO%	%
Monocytes percentage	MONO%	%
Eosinophils Percentage	EO%	%
Basophils Percentage	BASO%	%
A / G Index	A/G	-
Alanine aminotransferase	ALT	UI/L
Aspartate aminotransferase	AST	UI/L
Glutamyltranspeptidase	GGT	UI/L
Alkaline phosphatase	ALP	UI/L
Creatinine	CREA	mg/dL
Total Protein	TP	g/dL
Albumin	ALB	g/dL
Glucose	GLU	mmol/L
Cholesterol	CHOL	mg/dL
Total Bilirubin	TB	mmol/L
Direct Bilirubin	BIL-D	mmol/L
Pancreatic amylase	AMY-P	U/L
Calcium	CA	mg/dL
Cholinesterase	CHE	U/L
Phosphorus	PHOS	mg/dL
Triglycerides	TRIG	mg/dL
Urea	UR	mg/dL
Uric Acid	UA	mg/dL

Table 2: *Hematological and Serum biochemical parameters analyzed in the study.*

Macroscopic and histopathological evaluation

The macroscopic observation of all organs was performed during necropsy. The histopathological evaluation was performed in the following samples adrenal glands, thymus, lungs (with bronchi and bronchioles), heart, liver, spleen, kidneys, testes, prostate, brain, pituitary

gland, uterus (cervix and oviducts), ovaries, pancreas, thyroid and parathyroid gland, lymph nodes, mammary glands, salivary glands, parathyroid gland, skeletal muscle, lymph nodes, mammary glands, salivary glands, bone marrow, trachea, aorta, esophagus, stomach, small and large intestine, ureters, urinary bladder, epididymis, seminal vesicle, vagina, peripheral nerves, eyes and optic nerve, spinal cord, larynx, tongue and the site of application (skin and nasopharyngeal mucosa samples). The total and relative weights of all organs were measured and determined, respectively according to [12]. Briefly, samples were placed in 4% neutral formalin and processed following the kerosene embedding method [13] to be stained with Eosin-Hematoxylin and observed under a Carl Zeiss simple microscope (VistaVision, MO 000004, Zeiss, Germany) at 40x and 100x magnification [14]. Photomicrographs were taken with a Canon Power Shot digital camera (Canon, Japan). For the morphometric study of spleen, all monkeys in each experimental group were selected. Photographs of the organ were digitized with a digital camera (Nikon) at an average distance of 15 cm. A digital analysis of the 100x image of histological smears was performed using following morphometric parameters that quantify the size and shape of germinal centers (where diameter length and perimeter were taken into account). Image software version 1.36b [15] (Wayne Rasband, National Institutes of Health, USA), available at http://rsbweb.nih.gov/ij/index.html, was used. A morphometric study of the spleen was also performed in two animals of the Placebo, High Dose and Reversion groups; for which the area and perimeter of the periarteriolar zone of the lymphoid follicles of 3 fields (100X magnification) of the organ were determined, using the ImageJ®1.43u program [16]. The microscopic analysis of administration site, and evaluation as irritability was done according to the ISO/WD 10993-10 standard [17].

Statistical analysis

Statistical analysis was performed using the SPSS Statistics version 26 program for Windows (2020). All studied parameters were reported as means or medians, standard deviation and ranges according to age and sex of studied monkeys. The Shapiro-Wilk test was applied to verify the assumption of normality and the Levene test was applied to verify date variance homogeneity. The one-way ANOVA test was used when data fulfilled either assumptions or the Kruskal-Wallis test when data, even after transformations, did not meet any of these mathematical conditions. For the analysis of the differences between the beginning and the end of the study in each group, the Student's test was used for the data that appeared normal distribution, and the Wilcoxon test for the data that did not appear this distribution. The Mann-Whitney test was applied to determine the significance of the presentation of the most notable microscopic lesions. The significance level (α) used in all analysis was 0.05%.

Results

Clinical and behavior analysis results

None monkey died during the trial and no clinical or behavioral alterations were detected in the studied monkeys. Variation in the body weight was not detected between mean values obtained at the beginning and at the end of the study (Table 3). No affectations were detected throughout the trial in the water and food avidity and behavioral variables, as well as the rectal temperature of all monkeys was within the physiological range for the specie (38.0 - 39.5°C). With respect to males, no significant differences were detected among experimental groups at any time ($P > 0.05$). Regarding the analysis over time, significant differences were evidenced among different times only in High-Dose/Reversion groups ($P = 0.032$). In females, rectal temperature only showed significant differences ($P < 0.05$) among results of the Low-Dose group, Placebo group and High-Dose/Reversion groups on day 8 of the study. During the rest of the study, no differences were detected among experimental groups or among monkeys in the same experimental group ($P > 0.05$).

	Males			
	Placebo	Low-Dose	High-Dose	Reversion
	Mean	Mean	Mean	Mean
Day 0	4.11a	4.91a	5.62a	4.06a
Day 28	4.67a	4.60a	5.71a	3.98a
Day 56	-	-	-	4.09a
	Females			
Day 0	3.07a	3.41a	3.64a	2.90a
Day 28	3.14a	3.35a	3.47a	2.76a
Day 56	-	-	-	2.97a

Table 3: *Mean of corporal weight (kg) in males and females.*

Different letters means statistical differences among animals of same groups (P < 0.05).

Hematological parameter determination results

The hematological parameter analysis evidenced that, in both genders, WBC, MONO%, BASO%, LYMPHO%, NEUTRO%, EO%, RBC, HGB, HCT, MCV, MCH and MCHC did not shown variations among values measured before starting Jusvinza administration and at the end of the study. No significant differences were detected when results obtained of different experimental groups were compared (P > 0.05) (Table 4 and 5). Conversely, PLT and EO% results revealed a slight decrease in all experimental groups. Regarding comparison among experimental groups, in the case of males, significant differences were only detected in the case of PLT (P = 0.017), where monkeys of the Placebo group showed lower values than those of values determined in the High-Dose and Reversion groups (Table 4). In the analysis of hematological parameters of females, monkeys of the Low-Dose group showed significant lower LYMPHO% values (P = 0.017) than those measured in the High-Dose and Reversion groups. Likewise, NEUTRO% values were significantly lower in the High-Dose and Reversion groups compared to the Low-Dose group. In addition, females belonged to the High-Dose and Reversion groups showed lower platelet values than values of monkeys of the Low-Dose group (P = 0.006) (Table 5).

	Physiological range	Placebo 28 days	Low-Dose 28 days	High-Dose/Reversion 28 days	Reversion 56 days
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
WBC	7.05 - 14.68	13.13 ± 4.93a	11.43±2.77a	11.96 ± 3.80a	9.75 ± 0.21a
LYMPHO %	35.96 - 85.68	43.00 ± 13.45a	49.67 ± 13.28a	68.2 ± 19.77a	54.00 ± 9.90a
MONO %	2 - 3.99	0.00a	1.00±1.73a	0.60± 0.86a	0.00 ± 0a
NEUTRO %	7.59 - 56.32	56.67 ± 14.01a	49.00 ± 12.17a	31.2 ± 20.46a	46.00 ± 9.90a
EO %	0.32 - 8.32	0.33 ± 0.58a	0.00 ± 0a	0.00 ± 0a	0.00 ± 0a
BASO %	0.02 - 0.68	0.00 ± 0a	0.33±0.58a	0.00 ± 0a	0.00 ± 0a
RBC	4.67 - 6.30	3.89 ± 0.21a	3.58±0.13a	3.506 ± 0.34a	3.73 ± 0.27a
HGB	11.32 - 15.07	13.30 ± 0.75a	11.83 ± 0.47a	12.16 ± 1.12a	13.85 ± 0.64a
HCT	37.32 - 48.44	45.67 ± 2.87a	41.03 ± 1.12a	41.4 ± 4.23a	47.80 ± 2.12a
MCV	70.48 - 85.94	117.00 ± 1.73a	114.67 ± 1.15a	118.2 ± 2.77a	128.50 ± 3.54a
MCH	21.72 - 26.39	34.20 ± 0.20a	33.10 ± 1.08a	34.72 ± 0.85a	37.15 ± 1.06a

MCHC	28.61 - 32.93	29.17 ± 0.25a	28.87 ± 0.75a	29.4 ± 0.46a	28.90 ± 0a
PLT	236.2 - 665.7	147.33 8.14a	278.67 ± 155.09ab	298.4 ± 80.90b	230.00 ± 18.38b
A/G	1 - 7	1.40 ± 0.17a	1.37 ± 0.06a	1.30 ± 0.16a	3.35 ± 3.18a
ALT	37 - 99	43.33 ± 25.32a	55 ± 21.66a	56.60 ± 39.74a	31 ± 8.49a
ALB	3.03 - 4.17	4.26 ± 0.23a	4.27 ± 0.19a	4.33 ± 0.17a	4.19 ± 0.17a
ALP	73 - 402	303 ± 139.12a	179.33 ± 93.07a	167.60 ± 67.15a	220 ± 83.44a
AMY-P	235 - 453	393.33 ± 159.95a	377.33 ± 88.49a	358.60 ± 114.96a	-
AST	32 - 62	32.67 ± 11.37a	71.33 ± 12.01b	43 ± 12.63ab	26 ± 12.73a
BIL-D	0.01 - 0.27	0.05 ± 0.01a	0.04 ± 0.03a	0.05 ± 0.03a	0.09 ± 0.01a
TB	0.02 - 0.57	0.08 ± 0.02a	0.06 ± 0a	0.11 ± 0.06a	0.18 ± 0.03a
CA	9.3 - 10	9.98 ± 0.26a	9.89 ± 0.23a	9.52 ± 0.25a	2.40 ± 0.09a
CHE	109.6 - 16553	11840.3 ± 2413.73a	13569.7 ± 4053.52a	10991.6 ± 3175.41a	9405 ± 731.15a
CHOL	100 - 180	87.82 ± 15.81a	94.23 ± 11.81a	108.29 ± 14.50a	139.13 ± 22.87a
CREA	0.8 - 1.14	0.63 ± 0.03a	0.59 ± 0.04a	0.59 ± 0.12a	0.66 ± 0.05a
GGT	23 - 97	88.33 ± 30.09a	70.33 ± 29.16a	80.20 ± 38.23a	87 ± 43.84a
GLU	81 - 123	76.02 ± 17.84a	74.11 ± 8.35a	64.93 ± 12.29a	67.65 ± 8.80a
PHOS	3.40 - 7.60	4.51 ± 1.29a	3.49 ± 1.53a	5.28 ± 1.13a	5.76 ± 0.75a
TP	5.9 - 7	7.33 ± 0.43a	7.41 ± 0.37a	7.67 ± 0.21a	7.69 ± 0.56a
TRIG	19 - 52	33.18 ± 9.70a	50.17 ± 16.38a	43.47 ± 32.17a	39.33 ± 8.05a
UA	0.1 - 0.3	0.02 ± 0.03a	0.02 ± 0.02a	0.00 ± 0.01a	0.02 ± 0.02a
UR	28 - 50	56.59 ± 5.39ab	69.16 ± 2.94b	51.55 ± 8.02a	40.28 ± 14.32a

Table 4: *Mean, standard deviation and physiological range of hematological and serum biochemical parameters in males. Different letters means statistical differences among experimental groups (P < 0.05).*

	Physiological range	**Placebo 28 days**	**Low-Dose 28 days**	**High-Dose/Reversion 28 days**	**Reversion 56 days**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
WBC	5.64 - 12.41	7.80 ± 2.44a	9.53 ± 1.99a	8.16 ± 2.31a	8.15 ± 2.05a
LYMPHO %	35.18 - 84.57	48 ± 9.54ab	41 ± 8.89a	68 ± 12.26b	50 ± 15.56a
MONO %	0 - 2.56	0 ± 0a	0.67 ± 1.15a	0.2 ± 0.44a	0 ± 0a
NEUTRO %	17.56 - 56.75	51.33 ± 10.12ab	61.67 ± 13.05b	31.2 ± 12.09a	50 ± 19.80ab
EO %	0.51 - 10.07	0.67 ± 0.58a	0 ± 0a	0.4 ± 0.89a	0 ± 0a
BASO %	0.16 - 1.98	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
RBC	4.64 - 6.24	3.50 ± 0.21a	3.23 ± 0.17a	3.48 ± 0.23a	3.71 ± 0.02a
HGB	11.01 - 14.86	11.77 ± 0.87a	10.80 ± 0.85a	11.14 ± 0.73a	13.45 ± 0.21a
HCT	36.1 - 48.29	40 ± 2.76a	37.33 ± 2.56a	38.5 ± 2.67a	46.50 ± 1.70a
MCV	70.48 - 84.75	114.67 ± 2.08a	115.67 ± 4.16a	110.4 ± 2.79a	125.50 ± 3.54a
MCH	21.61 - 25.95	33.67 ± 0.71a	33.57 ± 1.53a	32.04 ± 1.02a	36.40 ± 0.42a

MCHC	28.49 - 32.84	29.40 ± 0.20a	29 ± 0.26a	29 ± 0.63a	28.95 ± 0.64a
PLT	205.9 - 661.3	268.33 ± 65.92ab	354.33 ± 34.67a	219.8 ± 55.17b	218.50 ± 38.89
A/G	1 - 6	1.47 ± 0.15a	1.17 ± 0.15a	1.36 ± 0.19a	1.20 ± 0.14a
ALT	45 - 168	78.67 ± 41.10a	101.33 ± 48.79a	70.60 ± 67.14a	29.50 ± 9.19a
ALB	3 - 5	4.07 ± 0.25a	4 ± 0.26a	3.94 ± 0.14a	4 ± 0.05a
ALP	41 - 456	100.33 ± 12.34a	114 ± 27.73a	156 ± 74.78a	201 ± 106.07a
AMY-P	269 - 328	313.67 ± 30.50a	456 ± 198.28a	307.60 ± 48.60a	-
AST	26 - 80	46.33 ± 14.57a	85 ± 40.73a	42.40 ± 17.05a	36.50 ± 4.95a
BIL-D	0.01 - 0.1	0.06 ± 0.01a	0.05 ± 0.01a	0.04 ± 0.01a	0.05 ± 0.01a
TB	0.02 - 0.53	0.10 ± 0.02a	0.10 ± 0.06a	0.28 ± 0.52a	0.13 ± 0.13a
CA	8 - 10	9.11 ± 0.34a	10.01 ± 0.40a	9.36 ± 0.45a	2.42 ± 0.13a
CHE	754 - 14858	11751.3 ± 1978.30a	16726.3 ± 557.37a	14560.2 ± 2694.53a	15681 ± 5123.29a
CHOL	96 - 78	97.95 ± 18.65a	84.75 ± 40.73a	105.82 ± 21.91a	132.09 ± 13.99a
CREA	0.6 - 0.9	0.48 ± 0.09a	0.51 ± 0.06a	0.46 ± 0.08a	0.64 ± 0.12a
GGT	25 - 95	55.67 ± 27.32a	50 ± 20.07a	44.20 ± 9.68a	51.50 ± 9.19a
GLU	75 - 113	55.25 ± 9.71a	58.25 ± 9.96a	54.41 ± 7.53a	66.84 ± 4.58a
PHOS	4.30 - 8.30	3.98 ± 0.69a	2.76 ± 1.03a	3.13 ± 1.27a	4.46 ± 0.83a
TP	5.4 - 7	6.93 ± 0.67a	7.38 ± 0.10a	6.85 ± 0.22a	7.39 ± 0.42a
TRIG	45 - 69	29.46 ± 6.21a	39.83 ± 2.30a	42.43 ± 8.86a	45.30 ± 5.24a
UA	0.1 - 0.3	0.03 ± 0.03a	0.03 ± 0.02a	0.01 ± 0.01a	0.01 ± 0.01a
UR	19 - 40	52.16 ± 11.15a	74.31 ± 15.29a	50.67 ± 6.76a	46.77 ± 14.91a

Table 5: *Mean, standard deviation and physiological range of hematological and serum biochemical parameters in females. Different letters means statistical differences among experimental groups (P < 0.05).*

Serum biochemical parameter determination results

The analysis of AG, ALP, ALB, BIL-D, GGT and CA of all monkeys showed values within the physiological range of the specie, and no statistical differences were detected between data obtained at the beginning and at the end of the study were compared (P > 0.05). On the other hand, despite study started with values within the physiological range for GLUC, TP, AST, CREA, UA and UR parameters, an increase and/or decrease in parameter values was evidenced in relation to the physiological range in an independent treatment group manner (Table 4 and 5). For TRIG, CHOL, PHOS and BIL-T of the Reversion group, mean values were out of the physiological range in the evaluations performed immediately after the administration period, although once the Reversion period was over, values returned within the physiological range. The pancreatic amylase (AMY-P) analysis revealed values (456 ± 198.28 U/L) above the physiological range (235 - 453 U/L) in females of the Low-Dose group (Table 4 and 5). Statistical differences were found only in the UR and AST parameters (P < 0.05) between different experimental groups. Significant differences were estimated between the final UR of the High-Dose group and the Low-Dose group (P = 0.013), while in AST analysis, the Placebo group showed statistical lower values (P = 0.016) in comparison with the Low-Dose group (Table 4). On the contrary, in females, no differences (P > 0.05) were detected among studied parameter values (Table 5).

Macroscopic and microscopic analysis results

The most important alterations were detected at the adrenal glands and spleen administration sites. Slight hemorrhages were observed in the subcutaneous cellular tissue of the skin corresponding to the administration site as well. Hemorrhages were observed in 3 monkeys (50%) of the Placebo group, and in one monkey treated with the high dose of Jusvinza (0.7 mg/kg). The adrenal glands showed a decrease in the size in one monkey of the Placebo group and in two monkeys of the Low-Dose group (33.33%). In the spleen, although results obtained during the morphometric study of lymphoid sheath zone (PALS) of lymphoid follicles; significant differences among values of experimental groups, during the macroscopic observation were not detected. A moderate splenomegaly was only detected in two monkeys administered with the high dose of Jusvinza. This finding was later complemented by a microscopic analysis corroborating hyperplasia of the lymphoid follicles in the spleen, where cell proliferation was also detected in the periarteriolar lymphoid sheath zone (PALS) of the lymphoid follicles, with formation of large germinal centers (Image 1), in two monkeys (33%) treated with the low dose and in three monkeys (50%) treated with the higher dose of Jusvinza. Adhesions in the apical lobe of the lungs and discrete pneumonic foci were observed in one female of the Placebo group. The absolute weight analysis only showed significant differences ($P < 0.05$) between lungs of females in the Low-Dose group ($P = 0.017$) and in the Placebo group ($P= 0.015$). No significant differences in the relative weight of all monkey organs were determined ($P > 0.05$). Nevertheless, additional findings were identified in the digestive system. For instance, hyperplasia in Gut-Associated Lymphoid Tissue (GALT) was detected in monkeys of all experimental groups, with a great incidence in the stomach (Image 2). Besides, this type of hyperplasia was also detected in the small and large intestines (Image 3). Hyperplasia in the stomach occurred in two monkeys (33%) of the Placebo and High-Dose groups, in three monkeys (50%) of the Low-Dose group and in all monkeys of the Reversion group, after 28 days of the last Jusvinza administration. In the thymus, a partial loss of parenchymal tissue was observed. This issue was characterized by a moderate decrease of lymphoid cells in the cortex, and had incidence in one monkey of the Placebo, Low and High-Dose groups, and in three monkeys (75%) of the Reversion group. Hepatocytes with a clear cytoplasm were visualized diffusely in all assessed monkeys. Likewise, the presence of focal steatosis and in the periphery of the central vein was detected in one (16.66%) monkey in each experimental group, a finding characterized by the presence of multiple optically empty vacuoles in the cytoplasm of hepatocytes, with macro and micro-vesicular character.

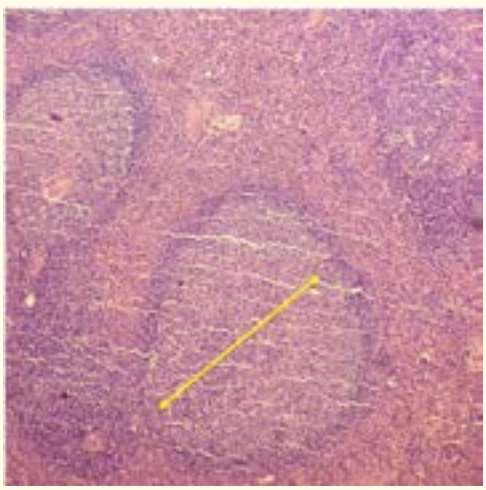

Image 1: *Hypertrophy of germinal centers in spleen.*

High-Dose group.
Animal 0303.
HE 100X.

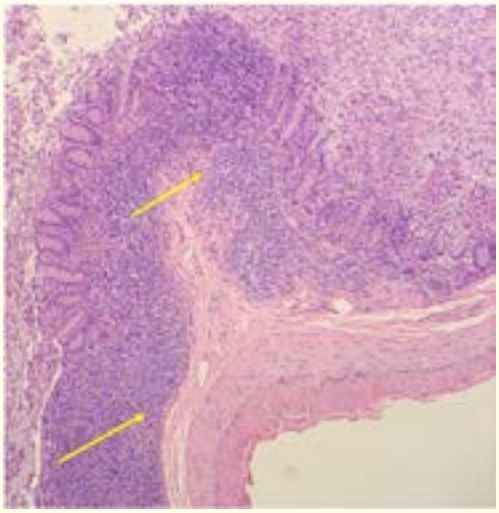

Image 2: *GALT hyperplasia in stomach.*

High-Dose group.
Animal 0302.
HE 100X.

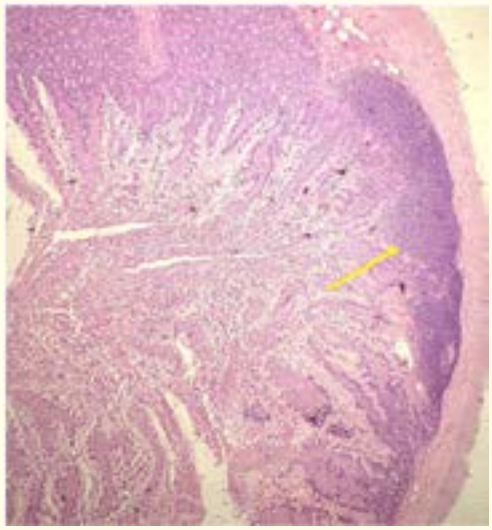

Image 3: *GALT hyperplasia in the colon.*

Reversion group.
Animal 0401.
HE 100X.

Discussion and Conclusion

The complexity of immune and physiologic parameters in NHP is an indirect mirror of the complexity of human systems as well [18]. Therefore, NHP are excellent animal models for conducting toxicological studies in life-science. In comparison with other model animals such as rodent or canine species, NHP immune system shows an extraordinary homology with the human immune system [19]. Due to these similarities, NHP models provide a critical biomedical resource for accelerating preliminary safety testing of vaccines and other pharmaceutical interventions that cannot be ethically evaluated in human subjects prior to clinical trials such as Jusvinza [18].

In general, Jusvinza is an altered peptide ligand (APL) derived from a CD4+ T cell epitope of the human heat-shock protein 60 (HSP60), an autoantigen involved in the RA pathogenesis [20]. This mechanism suggested the possibility of using Jusvinza in the treatment of patients affected by the cytokine storm that occurs in patients affected by COVID-19. Thus, results obtained with Jusvinza application were compared with the non-clinical results obtained with other steroidal and non-steroidal anti-inflammatory drugs used in the treatment of patients with RA and COVID-19 in severe and critical condition.

During this study, no clinical alterations were detected in the studied *Macaca fascicularis* monkeys, which allowed stated that Jusvinza does not affect monkey clinical status. However, the administration of CI-986, a novel anti-inflammatory compound provoked emesis and diarrhea in *Macaca fascicularis* monkeys, which an increased incidence and severity at the higher doses 500 mg/kg/day [21] and nephrotoxicity [22]. These differences could be attributed to the doses and the different nature of compounds under study, although both have anti-inflammatory effects.

The body weight is an indirect indicator of animal welfare regularly measured during preclinical safety studies [23]. Therefore, the absence of decreases in the body weight detection in the studied monkeys lead to the conclusion that Jusvinza does not produced health alterations or toxicity in the *Macaca fascicularis* monkeys. Conversely, the oral administration of Lornoxicam, a non-steroidal anti-inflammatory compound, whose mechanism of action is basically related to the inhibition of prostaglandin synthesis in doses of 1 - 2 mg/kg/day in *Macaca fascicularis* monkeys caused a decrease in the body weight [24].

With respect to hematology parameters, the administration of Naproxen (a no steroid anti-inflammatory drug) by oral route in *Macaca fascicularis* monkeys provoked decreasing in values of hematocrits in monkeys that received 44 mg/kg [25]. A decrease in the total protein concentration and a not regenerative anemia was also detected. However, in the Jusvinza case, variation was only detected in PLT values, although, the slight variation returned to normal levels during the reversion period. The rest of hematological parameters did not shown variations, which allows inferring that Jusvinza, in the administered doses, despite being administered intravenously, does not produce severe effects on studied hematological parameters.

On the other hand, the analysis of the serum biochemical parameters evidenced that most biochemical parameters studied maintained values within reference ranges, demonstrating Jusvinza does not affect the most important biochemical parameters and cellular metabolism. In specific terms, in the case of AST and UR, the absence of differences detected among values obtained in the High-Dose and Placebo groups corroborated that these differences cannot be attributed to the Jusvinza effect. The very high AMY-P values measured in females of the Low Dose group was an indicator of pancreatic damage [26], but it also indicates that this damage was not related with Jusvinza action mode. Conversely, the administration of diclofenac potassium (a no steroid anti-inflammatory drug) in a dose of 100 mg/kg/day has been associated with a decrease in the total protein, albumin, albumin/globulin ratio, AST, and ALT [27]. This difference can be most likely explained by the low dose of Jusvinza administered and by the different mechanisms of action of this anti-inflammatory drug.

In regards to another mandatory study that has to be carried out in preclinical studies (analysis of the absolute weight of organs) [28], results revealed a difference in the absolute weight of organs without biological significance in Jusvinza evaluation. The observed differ-

ences are more associated with disparity in weight and age of monkeys. On the other hand, the relative weight of organs showed a close relation with the age [26], but not with the action mode of Jusvinza. In contrast, the administration of only two injections of dexamethasone in doses of up to 0.7 mg did not generate alterations in the weight of the organs of *Macaca fascicularis* monkeys [29], although this could be due to the low dosage and frequency administered, because other authors reported a significant reduction in thymus, spleen, and adrenal weights after treatment with dexamethasone [30].

Moreover, findings detected at the administration site of monkeys of the Placebo and High-Dose groups, with higher incidence in the Placebo group evidenced that these findings were not related with Jusvinza. It might be, probably, associated to the mechanical trauma produced by the needle in the inoculation process [31] or with the volume administered, observing a very similar frequency of the sign between High-Dose and Placebo groups, administered with the same volume, which did not show a toxic effect, since a total recovery was observed in monkeys of the Reversion group. Furthermore, issues observed in the microscopic analysis of the administration site allowed concluding that no signs of local intolerance were observed after the intravenous administration of Jusvinza or in Placebo group in 100% of *Macaca fascicularis* monkeys, demonstrating this product does not produce irritability and has a high tolerability showing a total recovery at macroscopic and microscopic level. However, administration of diclofenac sodium by subcutaneous route provoked reddening at the injection site in females and a black/red area around the injection site vein by intravenous route [27].

Non-steroid anti-inflammatory drug administration in monkeys may induce mild hepatic changes, characterized primarily by increases in liver enzymes without clinical signs or hepatic dysfunction [32]. In addition, the long-term use of nonsteroidal anti-inflammatory drugs has been associated with toxic effects in multiple organ systems, including the gastrointestinal tract and kidneys [33,34]. The most common adverse effects of ibuprofen are gastrointestinal irritation and ulceration [35]. On the other hand, the kidneys of *Macaca fascicularis* monkeys, which received methotrexate, revealed crystalline-like deposits of methotrexate within the lumen of renal tubules [36]. In the case of Jusvinza, despite highly significant evidence of GALT hyperplasia in the digestive system, no significant differences were found among experimental groups.

Although, results obtained in the biodistribution study, where after a single intravenous and intradermal administration of Jusvinza in Lewis rats, by both routes, after 4 hours and until 24 hours, the highest values were also detected in the stomach and small intestine [37], the presence of lymphoid hyperplasia in the digestive system of animals of all the experimental groups and the existence of normal abundant lymphoid tissue in the morphology of the gastrointestinal tract [38] could indicate that lymphoid hyperplasia, more prominent in the stomach and intestine of the animals of this study, is not related to the effect of Jusvinza, and this findings not affect the safety of Jusvinza.

The white pulp of spleen is mainly composed by T-lymphocytes and constitutes the site where the interdigitating dendritic cells process antigens, presenting associated to their MHC molecules (class II) to resting T-lymphocytes [39]. In this regards, the hyperplasia of the lymphoid follicles of the spleen, detected in monkeys of the High-Dose groups, can be a consequence of administration of Jusvinza, and these effects were totally eliminated once administration of the product was suspended.

The findings detected in the adrenal glands showed that there was no relationship between the decrease in their volume and Jusvinza administration. In NHP species used in toxicological studies, it is usual detecting similar pathologies to those detected in the adrenal gland analysis, even without the administration of a given particular substance [40].

The frequency of occurrence of pulmonary findings in this study also showed that changes were not related to Jusvinza administration. These finding could be associated with the sustained presence of microbiological, physical or chemical agents that produce these morphological changes in the lung parenchyma [41]. On the other hand, with respect to the atrophies of the thymus, it is a physiological degenerative process that begins with the arrival of puberty and occurs individually in each animal since it depends fundamentally on the hormonal secretions that are produced with the arrival of this stage ending with death [42] thus it is not related with effect produced by Jusvinza.

The area observed of hepatocytes with a clear cytoplasm in the liver corresponds with a morphological characteristic that could be classified as normal, when it acquires a diffuse character. It can be associated with the mobilization of hepatic glycogen in hours of fasting [43], as established for the extraction of blood before euthanasia of monkeys [44]. As for hepatic steatosis, the fatty change was considered minimal and focal and includes several causes of this etiology, such as diets rich in fat, individual hormonal balance, peculiarities of metabolism and food consumption [45].

Finally, in humans, administration of Jusvinza in critically COVID-19 patients shown to have no effects on temperature [46] and also a LYMPHO% increases, a NEUTRO% decreases [47], a significant increase in TregCD4 + CD25 + Foxp3 cells levels [48] associated with normalization of lymphocyte cell counts were demonstrated [46]. These activated cells migrate to inflammation sites and cross-recognize wild-type epitope from HSP60, which is expressed on endothelial tissue, inhibiting autoimmune-damages in the endothelium provoked by SARS-CoV-2 infection. The expansion of Treg cells contributes to a resolution of hyperinflammation evidenced by a reduction of inflammation biomarkers and a positive outcome of COVID-19 patients [49]. These findings showed correspondence with results obtained in NHP, validating the *Macaca fascicularis* monkeys as an adequate animal model and the proper development of this toxicological study. Summarizing, given evidences obtained in this study, no alterations related to a possible toxic effect of Jusvinza in *Macaca fascicularis monkeys* were detected, allowing concluding that Jusvinza administered intravenously during 28 consecutive days, in the dose spectrum studied, is a safe product.

Conflict of Interest

Authors declare to have none conflict of interest to publishing this paper in scientific journals.

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