Research, Society and Development, v. 13, n. 3, e2713340644, 2024
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v13i3.40644

Study of the safety and therapeutic efficacy of multipotent adult progenitor cells in the treatment of feline asthma

Estudo da segurança e eficácia terapêutica das células progenitoras adultas multipotentes no tratamento da asma felina

Estudio de seguridad y eficacia terapéutica de células progenitoras adultas multipotentes en el tratamiento del asma felina

Received: 02/18/2023 | Revised: 12/06/2023 | Accepted: 03/07/2024 | Published: 03/10/2024

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Abstract
Feline asthma is a disease that is prevalent in young or middle-aged cats, with the main clinical signs being dry cough, mouth breathing, expiratory dyspnea, wheezing, cyanosis, crackles and weight loss. However, current therapeutic approaches have proven to be ineffective in preventing or reversing all pathological aspects of feline asthma. In this way, the development of new treatments for feline asthma that prove to be safe and effective are of fundamental importance for veterinary medicine. Stem cell therapy is presented to the market as a promising therapeutic approach since it has been shown to be safe and effective in the treatment of several diseases that affect both large and small animals. Therefore, this study aims to analyze the safety and efficacy of the treatment of feline asthma through stem cell transplantation, which mainly aims to obtain a methodology that allows the improvement of the quality of cats.
Keywords: Stem cells; Feline asthma; Cell therapy; Therapeutic safety; Therapeutic efficacy.

Resumo
A asma felina é uma doença que apresenta uma prevalência em felinos jovens ou de meia-idade tendo como principais sinais clínicos tosse seca, respiração oral, dispneia expiratória, sibilos, cianose, crepitações e perda de peso. Entretanto, as abordagens terapêuticas atuais vêm se mostrando pouco eficazes quanto a prevenção ou reversão de todos os aspectos patológicos da asma felina. Desta forma, o desenvolvimento de novos tratamentos para a asma felina que se mostrem seguros e eficazes são de fundamental importância para a medicina veterinária. A terapia com células-tronco se apresenta ao mercado como uma abordagem terapêutica promissora uma vez que vem se mostrando segura e eficaz no tratamento de diversas doenças que acometem tanto os grandes como os pequenos animais. Sendo assim, este estudo tem por objetivo analisar a segurança e eficácia do tratamento da asma felina por meio do transplante com células-tronco, o qual visa principalmente a obtenção de uma metodologia que possibilite a melhoria da qualidade dos gatos.
Palavras-chave: Células-tronco; Asma felina; Terapia celular; Segurança terapêutica; Eficácia terapêutica.

Resumen
El asma felina es una enfermedad prevalente en gatos jóvenes o de mediana edad, siendo los principales signos clínicos tos seca, respiración bucal, disnea espiratoria, sibilancias, cianosis, crepitantes y pérdida de peso. Sin embargo, los enfoques terapéuticos actuales han demostrado ser ineficaces para prevenir o revertir todos los aspectos patológicos del asma felina. De esta forma, el desarrollo de nuevos tratamientos para el asma felina que demuestren ser seguros y efectivos es de fundamental importancia para la medicina veterinaria. La terapia con células madre se presenta al mercado como un enfoque terapéutico prometedor ya que ha demostrado ser segura y efectiva en el tratamiento de varias enfermedades que afectan tanto a animales grandes como pequeños. Por ello, este estudio tiene

como objetivo analizar la seguridad y eficacia del tratamiento del asma felino mediante el trasplante de células madre, cuyo principal objetivo es obtener una metodología que permita la mejora de la calidad de los gatos.
Palabras clave: Células madre; Asma felina; Terapia celular; Seguridad terapéutica; Eficacia terapéutica.

1. Introduction

In recent years, veterinary medicine has evolved clinically and laboratorially, which has resulted in an increase in the life expectancy of both small and large animals. As a result of this increase in life expectancy, animals have suffered from diseases that were previously non-existent or of low incidence. These include osteoarthritis, kidney disease, dental problems, diabetes, hearing and visual loss.

Feline asthma has a prevalence of 1% to 5% of the domestic cat population, with young or middle-aged animals of the Siamese and Himalayan breeds being the most frequently affected (Gómez et al., 2012; Trzil, 2020). Feline asthma can have different etiologies such as allergic (cigarettes, disinfectants, perfumes, sprays, granules), parasitic, bacterial, mycoplasma or idiopathic. The clinical signs are dry cough, mouth breathing, expiratory dyspnea, wheezing, cyanosis, crackles and weight loss, which can vary according to the degree of bronchial involvement. In the initial stages, these signs may be mild and/or occasional, and may worsen as the feline's life.

Multipotent adult progenitor cells (MAPCs), also known in the literature as mesenchymal stem cells, are present in a quiescent stage in specific niches in each tissue and are responsible for ensuring tissue homeostasis throughout the animals' lives. CPAMs are characterized by being undifferentiated, spindle-shaped, long and flat cells, presenting a fibroblastoid morphology, the ability to adhere to polymeric surfaces, high proliferation potential and the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages (Santos, 2018a). Due to their potential for expansion *in vitro*, as well as the maintenance of their immunomodulatory, paracrine and differentiation properties, even after long periods of cryopreservation, CPAMs are a potential resource for both *in vitro* and *in vivo* assays.

Although the mechanisms involved in CPAM differentiation process *in vivo* have not yet been fully understood, it is known that CPAMs act by modifying the microenvironment through the secretion of paracrine and/or endocrine factors. These potentiate the tissue repair process and the reparative potential is attributed to their proliferative capacity, plasticity, cell signaling, production of biomolecules, growth factors and immunomodulators (Fu et al., 2019).

The aim of this study is to describe the safety and efficacy of treating feline asthma by transplanting allogeneic CPAMs derived from feline adipose tissue (CPAMs-TAF), with a view to improving patients' quality of life.

2. Methodology

Selection of adipose tissue donor animals

The 7 adipos tissue donor animals were selected from patient populations at veterinary clinics in the city of São Paulo, with the free and informed consent of their guardians. The CPAMs used in the present study were obtained from young, healthy animals up to six months old.

Selection of animals for the study

Twelve cats (two Persian, three Siamese, three Himalayan and four SRD), four intact males and eight intact females, ranging in age from 2 years and 7 months to 7 years and 3 months, weighing from 4 Kg to 6 Kg, affected by feline asthma, were submitted to the pilot study (Table 1). The pre-treatment patients were assessed using a liver profile (ALT and AF); renal function (urea, creatinine, albumin, sodium, potassium and calcium); erythrogram, leukogram, chest X-ray and abdominal ultrasound. Clinically, the patients were analyzed for symptoms of mouth breathing, dry cough, cyanosis, expiratory dyspnea,

wheezing, crackles and weight loss. Patients suffering from neoplasms, infections, hypertension or hypotension were excluded from the study.

Table 1 - The felines have been identified in the text as cats and their respective numbers. The table shows the characteristics of breed, sex, age (Y = years and M = months) and weight.

	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7	CAT 8	CAT 9	CAT 10	CAT 11	CAT 12
BREED	Persian	Persian	Siamese	Siamese	Siamese	Himalayan	Himalayan	Himalayan	SRD	SRD	SRD	SRD
SEX	Female	Male	Female	Fêmea	Male	Females	Females	Male	Females	Females	Females	Male
AGE	3 Y 2 M	2 Y 7 M	5 Y 2 M	6 Y 8 M	4 Y 7 M	3 Y 4 M	3 Y 6 M	7 Y 3 M	6 Y 5 M	5 Y 3 M	3 Y 1 M	7 Y 1 M
WEIGHT	2,7 kg	2,6 kg	1,7 kg	1,8 kg	3,1 kg	2,5 kg	2,7 kg	2,8 kg	2,8 kg	2,9 kg	2,6 kg	2,7 kg

Source: Authors.

Molecular Analysis

A blood sample was collected to analyze the presence of feline coronavirus (FCoV), feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), *Chlamydia psittaci* (CPS), feline herpes virus (FHV-1), feline calicivirus (FCV), *Haemobartonella felis* (HFE) and *Mycoplasma haemofelis* by amplifying fragments of their genomes using the polymerase chain reaction (PCR) method in extracted materials (RNA and DNA). RNAs were extracted with Trizol LS (Invitrogen®) and used for cDNA synthesis through reverse transcription with superscript II (Invitrogen®). DNAs were extracted using DNAzol (Invitrogen®). Two extracted samples, positive and negative controls, were submitted for each study (Santos et al., 2018b).

Isolation and characterization of CPAMs-TAF

The adipose tissue obtained at the time of castration was sent to the CELLTROVET Laboratory where it was washed in PBS 1x (phosphate buffered saline) to remove blood and debris. After washing, the tissue was kept for 30 minutes at 37ºC, 5% CO_2 in the presence of 0.075% collagenase type IV (Sigma-Aldrich®). 5 mL of basal medium was added, the supernatant removed and centrifuged for 5 minutes at 200xg. The precipitate was suspended and transferred to a 25 cm^2 culture bottle which was kept at 37ºC, 5% CO_2 for 48 hours in the presence of basal medium, when it was changed. Subsequent repiques were carried out by enzymatic action using 0.025% trypsin (Invitrogen®). The CPAMs were divided into aliquots of $2x10^6$, suspended in freezing medium (10% DMSO, 70% fetal bovine serum and 20% basal medium containing $2x10^6$ CPAMs) and stored in liquid nitrogen. The CPAMs were administered less than 1 year after storage. To be applied to felines, the cells were thawed and the cryopreservation medium removed (Santos, 2018a).

For the proliferative analysis, a colony of CPAMs-TAF was isolated and expanded until it reached 70% confluence in a 25 cm^2 plate. The cells were removed by enzymatic action (0.025% trypsin, Invitrogen®) and distributed in duplicates on 60 cm^2 plates at a concentration of 10^5 cells. After 48 hours of cultivation, the cells were removed and replated. The process was repeated until the 12^{th} passage. Cell viability was determined using *trypan blue* analysis, which showed a rate of 97% viable cells (Santos, 2018a).

The osteogenic potential of CPAMs-TAF was demonstrated using Von Kossa staining, after the cells had been kept in culture for 21 days in the presence of osteogenic differentiation medium (Dulbecco's Modified Eagle's Medium - Low Glucose, Invitrogen®), 1% 10^{-5} M dexamethasone (Sigma-Aldrich®), 1% 5 mM ascorbic acid (Sigma-Aldrich®), 10% fetal bovine serum (HyCloneTM) and 1% penicillin/streptomycin (penicillin G 10.000 IU/mL, streptomycin 10,000 µg/mL, Invitrogen®). The medium was changed every 3 or 4 days. On the 10^{th} day, 1% 200 mM β-glycerolphosphate (Sigma-Aldrich®) was added (Santos, 2018a). The cells were stained with 1% silver nitrate (Sigma) for 45 min under ultraviolet light, stained with 3%

sodium thiosulfate (Sigma) for 5 min and then contrasted with *Van Gieson*. At the end of this stage, the cells were washed thoroughly with ethyl alcohol and allowed to dry completely.

To analyze the adipogenic differentiation potential, the CPAMs-TAF were cultured in adipogenic differentiation medium (Dulbecco's Modified Eagle's Medium - Low Glucose, Invitrogen®), 10% fetal bovine serum (HyCloneTM), 1 mM dexamethasone (Sigma-Aldrich®), 100 mM indomethacin (Sigma-Aldrich®), 0.5 M isobutylmethylxanthine (Sigma-Aldrich®) + 10 µM insulin (Sigma-Aldrich®) and 1% penicillin/streptomycin (penicillin G 10.000 IU/mL, streptomycin 10,000 µg/mL, Invitrogen®), for 21 days and stained with *Oil Red O* (Sigma-Aldrich®). To do this, the cells were fixed with 4% paraformaldehyde for 30 minutes, washed with distilled water and stained with a working solution of 0.16% *Oil Red O* for 20 min (Santos, 2018a).

The chondrogenic differentiation potential was demonstrated by toluidine blue staining (Sigma-Aldrich®) after the CPAMs-TAF had been cultured for 21 days in the presence of the chondrogenic differentiation medium ((Dulbecco's Modified Eagle's Medium - High Glucose, Invitrogen®) supplemented with 1% fetal bovine serum (HyCloneTM), 6.25 mM insulin (Sigma-Aldrich®), 0.1 mM dexamethasone (Sigma-Aldrich®), 1 mM sodium pyruvate (Invitrogen®), 10 ng/mL TGF-β1 (R&D System, LGC Biotechnology) and 1% penicillin/ethreptomycin (penicillin G 10.000 IU/mL, streptomycin 10,000 µg/mL, Invitrogen®)). The cells were stained with 1% toluidine blue for one minute, washed with distilled water and then treated with a 70%, 95% and 100% ethyl alcohol solution wash, respectively (Santos, 2018a).

Transplantation of CPAMs-TAF

The patients underwent three transplants with the CPAMs-TAF with an average interval of 30 days between each infusion. A dose of $4x10^6$ CPAMs-TAF was used. The CPAMs-TAF were thawed in a water bath at 37°C for 2 minutes and transferred to a 15 ml falcon tube, adding saline solution in a 1:1 ratio. The cell concentrate was homogenized and centrifuged at 210xg for 5 minutes at room temperature. The supernatant was discarded and 3 ml of saline solution was added. The cell precipitate was resuspended and homogenized, then centrifuged at 210xg for 5 minutes at room temperature. This procedure was repeated 2 more times. The CPAMs-TAF were then resuspended in 5 ml of saline solution and homogenized for subsequent intravenous infusion. The applications were carried out intravenously (cephalic vein), without sedation. After thawing, the viability of the CPAMs-TAF was analyzed using *Triplan Blue* staining, which showed viability of over 90%.

Monitoring of animals undergoing cell transplantation with the CPAMs-TAF strain

The cats treated in this study underwent clinical and laboratory examinations. The tests were carried out on days 0, 15, 30, 45 and 60, as well as 180 days after the third transplant. The procedure was approved by CELLTROVET's Animal Use Ethics Committee under number 2/2022.

Eosinophilia analysis

After fasting overnight, six milliliters of blood were collected by jugular venipuncture and transferred to serum and sodium heparin tubes. The blood was left to clot for at least 15 minutes at room temperature and centrifuged at 1,720xg for 20 minutes. The serum was collected and the aliquots stored at -20°C. Eosinophilia was assessed on days 0, 15, 30, 45 and 60, as well as 180 days after the third transplant. The samples were Wright stained and subjected to a differential count of 200 nucleated cells. The data was reported as a percentage of eosinophils.

3. Results and Discussion

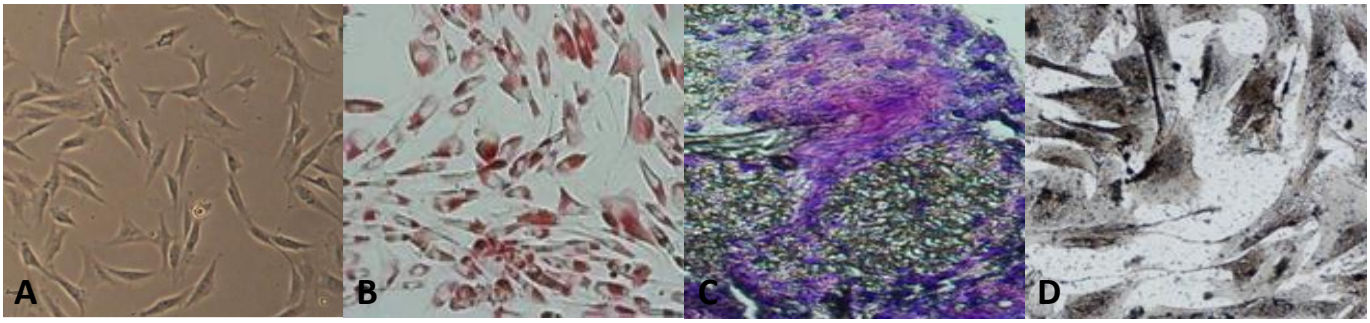
Cell therapy with stem cells has shown great promise in the treatment of various diseases affecting animals (Santos et al., 2023a). These include kidney disease (Santos et al., 2018b), hip dysplasia (Black et al., 2007), distemper (Santos et al., 2019), medullary aplasia (Santos et al., 2023b), keratoconjunctivitis sicca (Wei et al., 2022), feline gingivitis-stomatitis complex (Lembo et al., 2017) and tendon lesions (Depuydt et al., 2022). Stem cell transplantation aims, through its mechanisms of action, to cure or improve the quality of life of patients affected by numerous pathologies. The reparative potential of CPAMs-TAF allows them, once introduced into the body, to acquire both the morphology and functionality of any cell type, thus making it possible to restore damaged tissue.

Feline asthma is characterized by eosinophilia and airway hyperresponsiveness (AHR), as well as structural remodelling. This process is stimulated by an auxiliary immune response (Th)2 to inhaled aeroallergens (Grotheer et al., 2020; Vientós-Plotts et al., 2022; Agusti et al., 2023). Standard therapy consists of the application of glucocorticoids and bronchodilators, aimed at reducing the inflammatory process as well as airflow limitation. However, none of the current therapeutic approaches can prevent or reverse all the pathological aspects of asthma (Leemans et al, 2012; Chang et al, 2013; Grotheer et al, 2020).

The therapeutic use of CPAMs in the treatment of asthma has been extensively studied in murine models and is constantly associated with a reduction in asthmatic characteristics including airway eosinophilia, AHR and remodeling (Nemeth et al., 2010; Park et al., 2010; Choi et al., 2022). The process is based on the fact that CPAMs have immunomodulatory characteristics, helping to restore the Th1/Th2 balance in asthmatics, as evidenced by the decrease in Th2-related cytokines after treatment. In addition, CPAMs can promote immune tolerance through the process of proliferation of regulatory T cells (Tregs) and secretion of regulatory cytokines, such as interleukin (IL)-10 (Nemeth et al., 2010; Kavanagh & Mahon, 2011).

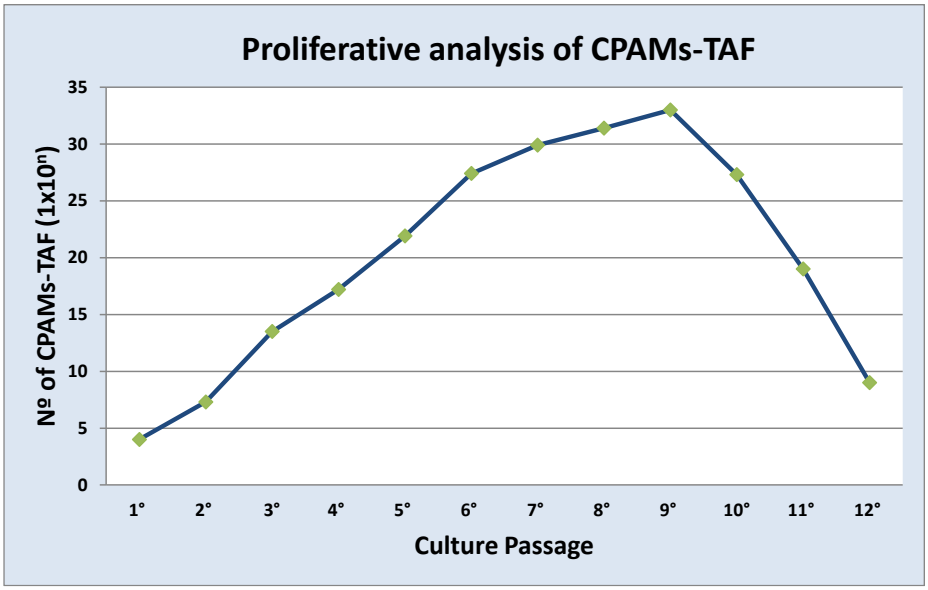
In this study, CPAMs-TAF were isolated from the adipose tissue of 7 young, healthy cats, establishing 7 distinct strains. At the CELLTROVET Laboratory, the CPAMs-TAF strains were characterized based on their ability to adhere to plastic, fibroblastoid morphology (Figure 1A), adipogenic differentiation capacity (showing the fat vesicles and lipid vacuoles characteristic of adipogenic cells) (Figure 1B), chondrogenic (showing the increased deposition of cellular matrix produced by the differentiated chondrocytes) (Figure 1C) and osteogenic (showing the presence of calcium deposits in the differentiated osteocytes) (Figure 1D), as well as high cell proliferation potential (Figure 2). Exponential growth was observed up to the 9th passage, after which there was a decay in the growth curve. When injected into nude mice, the 7 strains were unable to induce the generation of teratocarcinomas. The data obtained showed that all 7 strains had equivalent potential. As this was an allogeneic treatment, the donor animals were tested for positive reactions to DNA fragments of 185 bp and 410 bp (FCoV), 557 bp (FIV) 166 bp (FeLV), 167 bp (CPS), 173 bp (FHV-1), 205 bp (FCV), 170 bp (HFE) and 510 bp (mycoplasma), and the presence of the pathogens was not detected. In this way, possible transmission of them to recipient animals is avoided (Santos et al., 2018b).

Figure 1 - Fibroblastoid morphological aspect of stem cells isolated from feline adipose tissue (CPAMs-TAF)(A). Representative images of the adipogenic (B), chondrogenic (C) and osteogenic (D) differentiation of CPAMs-TAF. Objective 1x (A), 4x (B) and 20x (C, D).



Source: Authors.

Figure 2 - Representative graph of the CPAMs-TAF proliferative analysis. The growth of the cells was analyzed over a 48-hour interval between the repiques carried out using enzymatic action.



Source: Authors.

No início do estudo os 12 gatos apresentavam um quadro de ausculta torácica anormal, apresentando aumento dos ruídos respiratórios e sibilos, eosinofilia moderada (1.770 – 3.250/mm³) com o lavado broncoalveolar apresentando inflamação neutrófica e eosinofílica. As radiografias de tórax apresentaram padrões brônquicos com detecção de manguito peribrônquico. Estes dados demonstram que os 12 pacientes estavam sendo acometidos por um quadro de asma.

The 7 established strains were named CPAMs-TAF1, CPAMs-TAF2, CPAMs-TAF3, CPAMs-TAF4, CPAMs-TAF5, CPAMs-TAF6 and CPAMs-TAF7. The CPAMs-TAF1 strain was selected and transplanted intravenously via the cephalic vein into the 12 patients in order to test its safety and therapeutic efficacy. The transplants were carried out on days 0, 30 and 60, followed by fortnightly monitoring of the patients.

The intravenous route is characterized by being easy to perform, less invasive, with a high survival rate of the transplanted cells and less traumatic, allowing repeated applications with minimal side effects (Santos, 2017). CPAMs-TAF1 were administered in low concentrations and slowly, since studies suggest that pulmonary thromboembolism or infarcts can occur when a high cell concentration is administered rapidly intravenously (Prockop & Olson, 2007; Moll et al., 2012).

No adverse effects were identified from the CPAMs-TAF1 infusions, such as vomiting, nausea or changes in blood pressure, demonstrating that the transplant was well tolerated by the 12 cats. Follow-up of the patients did not reveal any type of pathology associated with the formation of abnormal tissue. Previous studies have shown that allogeneic CPAM transplants are safe in terms of rejection and do not require the use of immunosuppressive drugs (Assis et al., 2017; Santos et al., 2018b; Santos et al., 2019).

The first analysis of the patients was carried out 15 days after the first transplant when all the animals showed an improvement in appetite, weight gain and improved mood. In the assessment carried out on day 30, the date of the second CPAMs-TAF1 transplant, cats 2, 3 and 11 had normal chest auscultation, normalized respiratory sounds and wheezing, as well as a significant improvement in eosinophilia levels (890 - 1,470/mm³). Cats 1, 4, 5, 6, 7 and 10 already showed a significant improvement in chest auscultation and a reduction in respiratory noises and wheezes, as well as improvements in eosinophilia values (1,250 - 2,100/mm³). Cats 8, 9 and 12 still had significantly abnormal chest auscultation, breath sounds and wheezes and moderate eosinophilia (1,620 - 2,980/mm³).

In the analysis carried out 15 days after the second CPAMs-TAF1 transplant, cats 1, 2, 3, 5, 6, 7 and 11 had normal chest auscultation, normalized respiratory sounds and wheezing, as well as eosinophilia levels close to the reference values (670 - 820/mm³). Felines 4, 8, 9, 10 and 12 showed a significant improvement in chest auscultation and a reduction in respiratory noises and wheezes, as well as an improvement in eosinophilia levels (1,080 - 1,920/mm³).

On the assessment carried out on day 60, the date of the third CPAMs-TAF1 transplant, cats 1, 2, 3, 4, 5, 6, 7, 10 and 11 had normal chest auscultation, normal breathing sounds and wheezing, as well as normal eosinophilia levels (320 - 460/mm³). Felines 8, 9 and 12 showed a significant improvement in chest auscultation and a reduction in respiratory noises and wheezes, as well as a significant improvement in eosinophilia values (720 - 1,010/mm³).

180 days after the third application, the 12 cats were clinically and laboratory assessed. All the animals had normal chest auscultation, normal breathing sounds and wheezing and eosinophilia levels within the reference values (290 - 360/mm³).

4. Final Considerations

The data obtained in this study, through the transplantation of allogeneic CPAMs from feline adipose tissue, demonstrated the safety and therapeutic efficacy of CPAMs in the treatment of cats affected by feline asthma. The results showed that the therapy was effective regardless of the breed, sex or age of the cats. Based on the results presented, it is hoped that future research into the therapeutic potential of CPAMs in the treatment of diseases related to the respiratory tract of dogs and cats will lead to a better quality of life for pets.

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