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Assessment of metronomic chemotherapy–induced DNA damage in peripheral blood leukocytes from canine mammary cancer patients using the alkaline comet assay

Evaluación del daño en el ADN inducido por quimioterapia metronómica en leucocitos de sangre periférica de pacientes caninos con cáncer de mama mediante el ensayo cometa alcalino

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ABSTRACT

Mammary cancer is a disease that requires effective treatments. Conventional chemotherapy, while effective, often causes harmful side effects. In contrast, metronomic chemotherapy (mCHT), which involves the continuous administration of low doses of anticancer drugs, is presented as a less aggressive alternative. In this study, the genotoxic impact of treatment with Cyclophosphamide and Meloxicam under the mCHT approach was evaluated in ten canine (*Canis lupus familiaris*) patients with mammary carcinoma after undergoing mastectomy. The patients underwent monthly evaluations, including chest X–rays, blood tests, and the alkaline comet assay to measure genotoxic effects of the antineoplastic drugs. These results were compared with those of a group that received conventional chemotherapy. The results revealed that patients treated with mCHT experienced significantly lower levels of DNA damage compared to those who received conventional chemotherapy. Furthermore, DNA damage decreased over time during mCHT, suggesting that dogs may have developed tolerance to the treatment. Blood parameters remained stable in the mCHT–treated group, and X–rays showed no signs of recurrence or metastasis. All dogs survived during the one–year follow–up without mammary cancer recurrence. It is concluded that mCHT with Cyclophosphamide appears to be a less aggressive therapeutic option with a more favorable genotoxic profile in the treatment of mammary cancer in dogs.

Key words: Mammary cancer; metronomic chemotherapy (mCHT); cyclophosphamide; genotoxicity; mastectomy

RESUMEN

El cáncer de mama es una enfermedad que demanda tratamientos efectivos. La quimioterapia convencional, aunque eficaz, con frecuencia ocasiona efectos secundarios perjudiciales. En contraste, la quimioterapia metronómica (mCHT), que implica la administración continua de dosis bajas de fármacos anticancerígenos, se presenta como una alternativa menos agresiva. En este estudio, se evaluó el impacto genotóxico del tratamiento con ciclofosfamida y meloxicam bajo el enfoque de mCHT en diez pacientes caninas (*Canis lupus familiaris*) con carcinoma mamario después de someterse a mastectomía. Las pacientes se sometieron a evaluaciones mensuales, que incluyeron radiografías de tórax, análisis de sangre y el ensayo cometa alcalino para medir efectos genotóxicos del antineoplásico. Estos resultados se compararon con los de un grupo que recibió quimioterapia convencional. Los resultados revelaron que las pacientes sometidas a mCHT experimentaron niveles significativamente menores de daño al ADN en comparación con las que recibieron quimioterapia convencional. Además, se observó una disminución del daño al ADN con el tiempo durante la mCHT, lo que sugiere que las perras podrían haber desarrollado tolerancia al tratamiento. Los parámetros sanguíneos se mantuvieron estables en el grupo tratado con mCHT, y las radiografías no mostraron signos de recurrencia o metástasis. Todas las perras sobrevivieron durante el año de seguimiento sin recurrencia del cáncer de mama. Se concluye que la mCHT con ciclofosfamida parece ser una opción terapéutica poco agresiva con un perfil genotóxico más favorable en el tratamiento del cáncer de mama en perras.

Palabras clave: Cáncer de mama; quimioterapia metronómica (mCHT); ciclofosfamida; genotoxicidad; mastectomía

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INTRODUCTION

Mammary cancer is the most common type of cancer in women and the most common cause of cancer-related deaths Worldwide, being currently one of the most commonly diagnosed cancers and the fifth leading cause of cancer-related deaths, with an estimated 2.3 million new cases Worldwide [1].

Mammary cancer cells grow uncontrollably and break away from the primary tumor spreading through the bloodstream or lymphatic system to other parts of the body, where they can form new tumors or invade nearby tissues, a process known as metastasis [2, 3]. The treatment plans for mammary cancer may vary depending on the type and stage of the cancer, as well as the individual's overall health and preferences. The most common treatment options for mammary cancer include surgery, chemotherapy, radiation therapy, targeted therapy, immunotherapy, stem cell or bone marrow transplant, and hormone therapy [4].

Conventional chemotherapy is the use of cytotoxic drugs to target rapidly proliferating cells, particularly when these cells, such as cancer cells, are especially vulnerable due to abnormalities in the mechanisms that control adaptive responses to stress and cell death [5, 6, 7]. Metronomic chemotherapy (mCHT) is a new method to administer low-dose of anticancer drugs on a continuous and/or frequent regular schedule (such as daily or weekly), usually over a long period of time, with the advantage of causing fewer side effects than standard chemotherapy, rarely developing acquired drug resistance, and being cost-effective due to the lower dose used [8]. However, as it is novel, mCHT requires further research for a better understanding of the mechanisms of action, in particular with regard to the antiangiogenic effect, the immune response, the direct effects on cancer cells, Deoxyribonucleic acid (DNA) damage (genotoxic effects) and the identification of biomarkers that predict the response of the organism and mechanisms of resistance to this treatment modality [9].

Chemotherapeutics target rapidly dividing cancer cells by directly or indirectly inducing DNA damage [10]. However, antitumor drugs are indiscriminate and do not selectively damage the DNA of tumor cells [11, 12, 13]. Conventional chemotherapy can damage DNA in cancer cells resulting in mutations or genome instability, which is a key feature of both cancer and aging [12, 14]. When DNA is damaged, it may lead to (1) mutations, which promote cancer, or (2) reduced DNA replication, DNA and/or Ribonucleic acid (RNA) synthesis, cell-cycle halt, cellular senescence, or cell death, which hastens the aging process and reduces the functional capacity of cells or organs [12, 15].

The single-cell gel electrophoresis (SCGE) or alkaline comet assay, a rapid, visual and sensitive method for quantify and analyze DNA damage at the individual cell [16, 17, 18], has been used to investigate the DNA-damaging effects of anti-neoplastic drugs and radiation used during cancer therapy [19]. The alkaline comet assay has been successfully used to demonstrate substantial accumulation of fragmented DNA due to chemotherapy and has also been employed to assess pre- and post-treatment levels of *in vivo* DNA damage in peripheral blood leukocytes of cancer patients undergoing chemotherapy [20].

There are a variety of parameters for evaluating genetic damage by comet assay. The length of the tail, the amount of DNA in the tail, and the tail moment are the most often utilized parameters. As tail length does not frequently alter after the tail is formed, it can only be employed at low levels of DNA damage [21]. As the damage is increased, the tail's intensity follows suit. The amount of DNA in the comet tail, which has a linear relationship with the frequency of breaking, is another relevant statistic but the most helpful and commonly used parameter is the tail moment, which combines the tail length and tail intensity into a single number [16].

The use of animal models in human pathology research serves as a valuable tool for exploring disease mechanisms and testing the therapeutic effects of new and future drugs [22, 23, 24, 25]. Canine (*Canis lupus familiaris*) mammary tumors, bearing striking similarities to human breast cancer in terms of primary tumors, metastases, clinical presentation, and treatment approaches, make dogs an invaluable model for human cancer research [26, 27, 28, 29, 30, 31, 32, 33, 34]. Notably, conducting trials with novel antitumor therapies in pet dogs within a veterinary clinical setting enables the collection of serial biologic samples and facilitates the investigation of dose, schedule, and corresponding pharmacokinetic/pharmacodynamic relationships [35].

In this context, the current study aimed to assess DNA damage in female *Canis familiaris* with mammary neoplasms undergoing mCHT (Cyclophosphamide and Meloxicam) using a modified comet assay to detect DNA single-strand breaks (SSB) and double-strand breaks (DSB).

MATERIALS AND METHODS

Study population

The study encompassed a diverse group of 10 female dogs, that included both purebred and mixed-breed individuals. All subjects were diagnosed with various types of mammary carcinoma, such as papillary tubule, solid mammary, complex, and micropapillary. The selection criteria included grade 1 and 2 tumors with staging between 2-4, absence of metastasis history, suitability for surgery (mastectomy), and eligibility for mCHT.

Treatment regimen

After mastectomy, mCHT was administered orally for a duration of 3 months. Cyclophosphamide was used at a dose of 10 $mg \cdot m^2$ every 48 hours (h). Furthermore, as part of the adjuvant treatment, a dose of Meloxicam at 0.01 $mg \cdot kg^{-1}$ was administered every 24 h for a duration of three months.

Monitoring

The animals underwent monthly evaluations, which included ventrodorsal and laterolateral projections chest radiographs (x-ray univet 300 HF, SERIAL D.11.1513629.15.123. Multimage. Cavaria-Italy) for the detection of thoracic metastases and a comet assay to assess the genotoxic effects of chemotherapy. Furthermore, monthly analyses of hematological and biochemical parameters (TABLE I) were conducted both before and after chemotherapy to assess the general condition of the patients, identify potential side effects, and evaluate treatment response. The blood sample was obtained from the cephalic vein after shaving and disinfecting the area. A 1 ml sample was collected in an EDTA tube for hematology analysis, 2 ml in a heparin tube for the comet assay test, and 5 ml without anticoagulant for blood chemistry assessment.

Comparison group

To enable meaningful comparisons, it was incorporated data from an additional group of patients diagnosed with neoplasms. This

group received conventional chemotherapy consisting of intravenous Doxorubicin at a dose of 30 mg·m^2 over three cycles, each with 21 d intervals. This inclusion in the study ensures a basis for thorough comparative analysis.

Comet assay procedure

Blood samples were obtained from each patient using a syringe containing 0.10 mg of heparin. Each sample consisted of 10 uL of blood mixed with 160 uL of Low Melting Point Agarose at 37°C. The comet assay procedure followed the protocol detailed by Lu *et al*. [16]. After electrophoresis, plates were fixed with 2 mL of absolute methanol, stained with DAPI, and covered with coverslips.

Image capture

Comets were recorded using a 20X objective in an Olympus BX53 epifluorescence microscope (Olympus Corporation, Ishikawa, Japan), equipped with an Olympus DP73 digital camera coupled to CellSens Dimension Software (Olympus) for image acquisition. A minimum of 50 comets were analyzed for each individual.

Statistical analyses

Statistical analyses were performed following the recommendations described in Sokal and Rohlf [36]. To compare the data of the hematologic and biochemical parameters determined among periods of treatment, a one-way analysis of variance was used. Blood values that did not meet the assumptions of normality and homoscedasticity were transformed: Hematocrit (HCT) was transformed by the relation θ=arcsen p, where p is a percentage. For the discrete variables (erythrocytes, leucocytes HB, HCM, MCV), the root square transformation was used.

The image analysis was carried out using the ImageJ software [37] along with the OpenComet plugins [38]. These tools allowed for the determination of primary measurements, such as tail length (TL) and the tail DNA percentage (Tail DNA %) which were used to calculate the Tail Moment $\left(TM = \frac{Tail\ Length \times Tail\ DNA\ \%}{100}\right)$, as proposed by Tice *et al*. [39]. In particular, the (Tail DNA %) measure is recommended in the scientific literature, as it helps reduce variability in the results, as indicated by Kumaravel *et al*. [40].

Since comet assay data did not meet the assumptions of normality and homoscedasticity, the non-parametric statistical Mann–Whitney U test was used for comparing two groups, and the Kruskal–Wallis test was utilized for comparing multiple groups using Statgraphics Centurion.

RESULTS AND DISCUSSION

TABLE I presents the mean ± standard deviation for each parameter related to basic biometry and blood chemistry in the patients, covering their data from the study's initiation throughout the three-month treatment period. The analysis of variance demonstrated no significant statistical differences (P>0.05) with respect to treatment duration among the analytes.

Conventional chemotherapy, while effective in cancer treatment, can induce various side effects due to its impact on both cancerous and healthy cells. The adverse effects caused by intravenous administration of Cyclophosphamide in conventional chemotherapy include tachycardia, marked leukopenia, and hyponatremia [41], as well as hemorrhagic cystitis [42, 43].

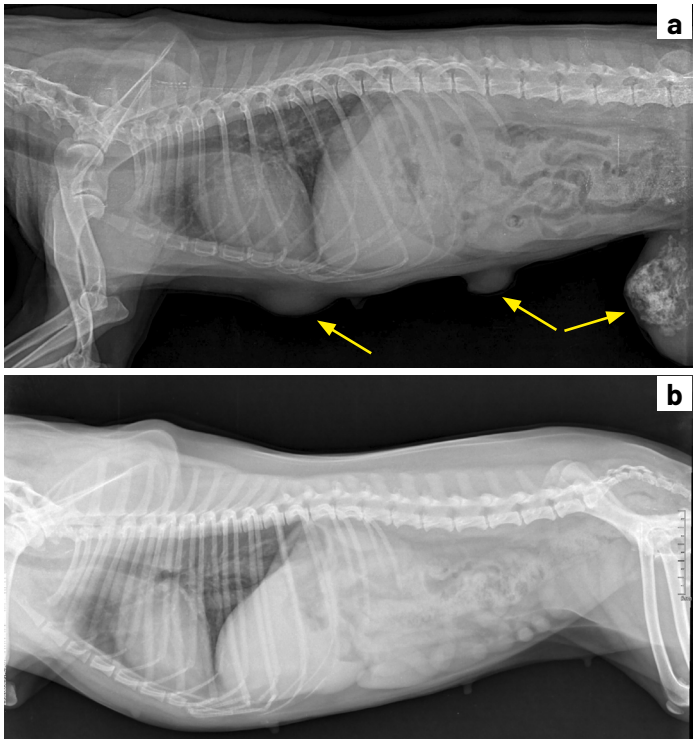
Cancer often leads to a deterioration in the patient's iron profile, potentially causing anemia. This condition can adversely impact the effectiveness of antineoplastic treatments and patient survival. To address the development of anemia, interventions such as iron and

TABLE I
Mean ± standard deviation of hematological and blood chemistry parameters in patients before (0) and after (1, 2 and 3 months) treatment with mCHT

	Treatment time (months)					
Parameter	**0**	**1**	**2**	**3**	***P***	**F**
Hematocrit (%)	42.90±3.12	42.60±3.80	41.60±3.3	42.70±3.06	0.8171	0.31
Total Solids (g·L^{-1})	86.40±9.28	86.60±6.75	89.10±10.06	85.50±7.47	0.8024	0.33
Hemoglobin (g·L^{-1})	127.00±9.69	131.20±12.25	121.20±9.94	128.50±7.76	0.1681	1.78
Erythrocytes (×10^{12}·L^{-1})	6.46±0.48	6.47±0.56	6.30±0.48	6.38±0.48	0.8646	0.24
VCM (fL)	66.66±0.08	66.63±0.14	66.64±0.13	66.67±0.00	0.5165	0.00
HCM (pg)	19.65±0.64	20.05±0.70	19.29±0.84	20.21±1.38	0.1388	1.95
CHCM (g·L^{-1})	296.09±11.93	305.33±9.29	292.29±14.99	303.20±20.72	0.1896	1.68
RDW (%)	14.87±0.81	14.88±0.78	15.22±1.02	15.10±0.79	0.7554	0.40
Leukocytes (×10^{9}·L^{-1})	11.60±2.56	12.25±5.59	11.12±4.56	15.00±5.93	0.3200	1.20
Platelets (×10^{9}·L^{-1})	281.80±69.62	299.60±92.58	312.83±95.56	343.90±207.48	0.6357	0.57
Urea (mg·dL^{-1})	35.30±9.47	40.40±8.03	34.80±12.95	32.30±9.45	0.3347	1.17
Creatinine (mg·dL^{-1})	1.02±0.18	0.92±0.21	1.03±0.19	0.89±0.26	0.3921	1.03
Total proteins (g·dL^{-1})	7.93±0.74	7.92±0.55	8.02±0.85	7.65±0.74	0.7059	0.47
GOT/AST (U·L^{-1})	77.10±75.36	51.00±19.91	38.30±19.33	55.20±24.24	0.3445	1.14
GPT/ALT (U·L^{-1})	57.00±41.12	61.10±20.70	35.00±19.71	170.30±262.02	0.1094	2.16
Ca (mg·dL^{-1})	8.70±0.75	8.72±1.02	8.3±0.60	8.08±0.68	0.2110	1.58

erythropoietin supplementation are employed [44]. Anemia can result from tumor-related factors, including inflammation, oxidative stress, and systemic metabolic changes in cancer patients, and it can be further exacerbated by the toxic effects of chemotherapy [45]. Notably, studies using Cyclophosphamide and Doxorubicin in conventional chemotherapy have reported a decrease in red blood cell count before and during treatment, with a more pronounced reduction during the second treatment cycle [46]. These findings underscore the importance of managing anemia to optimize both the efficacy of antineoplastic treatments and overall patient outcomes. Nonetheless, results here provided yielded no substantial disparities in any of the scrutinized blood parameters (TABLE I). What's particularly striking is that the metronomic regimen exhibited only a marginal effect on these metrics, implying that mCHT utilizing Cyclophosphamide might represent a milder treatment approach concerning its impact on blood parameters. This underscores the potential benefits of metronomic chemotherapy in ameliorating specific treatment-related adverse effects, ultimately contributing to improved patient comfort and treatment tolerability.

Radiological monitoring of patients with a diagnosis of mammary cancer before, during, and after metronomic chemotherapy treatment is displayed in FIG. 1 (ventro-dorsal projections are not included). Notably, the initial radiograph revealed well-defined neoplastic masses, indicative of the primary tumor lesions (FIG. 1a). Following tumor removal and three months of mCHT, subsequent radiographs showed no evidence of new opacities or nodules, suggesting a positive response to treatment in terms of controlling recurrence or metastasis (FIGS 1b, 1c, and 1d). The decision to perform a one-year follow-up radiograph (FIG. 1e) highlights the importance of long-term monitoring. These findings suggest that the treatment has been effective in controlling the disease and contributing to the overall well-being of the patient.



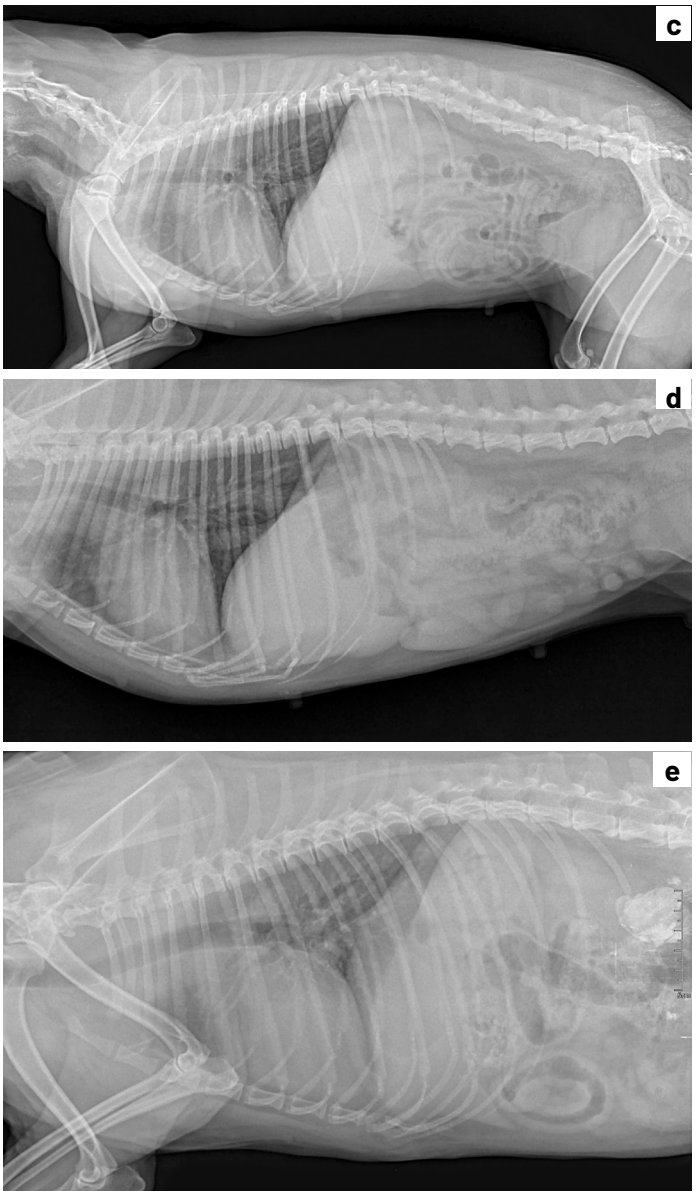


FIGURE 1. Radiographic images were assessed in laterolateral projections of the chest. Patient with three neoplastic mass (a) and first(b), second (c), and (d) thirds month of mCHT. (e) One year after completing mCHT treatment

Referring to the Comet assay, TABLE II illustrate the results of a comparative analysis of the assessed parameters before initiating metronomic therapy treatment and after three months of treatment. In all cases, the Kruskal-Wallis non-parametric test revealed highly significant differences ($P<0.001$) between the time points before and after treatment. The results of the Mann-Whitney test, examining Tail Length, Tail DNA %, and Tail Moment, are presented in TABLE III. In all cases, the variations in values between metronomic and conventional chemotherapy were found to be exceptionally statistically significant ($P<0.0001$).

MCHT and conventional chemotherapy represent distinct approaches to cancer treatment. Conventional chemotherapy employs higher drug doses at defined intervals, often based on bone marrow recovery, with the goal of directly targeting rapidly dividing

TABLE II
Comparison of Medians among treatment times (months) for Tail length (TL), Tail DNA % (T-DNA%) and Tail Moment (TM). Kruskal–Wallis Test Statistics (χ^2), *P*–value

Parameter	Treatment time (months)				Kruskal–Wallis test	
	0	1	2	3	χ^2	*P*–value
Tail Length	7.0	21.0	14.0	13.0	224.737	*P*<0.0001
Tail DNA %	8.02	12.1	11.49	8.23	137.705	*P*<0.0001
Tail Moment	0.976	2.553	2.301	1.035	137.666	*P*<0.0001
Sample Size	394	536	613	646		

TABLE III
Comparing Tail Length, Tail DNA %, and Tail Moment Between Metronomic and Conventional Treatments: Post hoc Wilcoxon–Mann–Whitney Test, *P*–value

Parameter	Metronomic	Conventional	W of Mann–Whitney	*P*–value
Tail Length	13.0	30.0	356347,0	*P*<0.0001
Tail DNA %	9.14	18.12	368349,0	*P*<0.0001
Tail Moment	1.283	5.942	368383,0	*P*<0.0001
Sample Size	1954	235		

tumor cells. In contrast, mCHT focuses on inhibiting angiogenesis within tumor tissues by administering chemotherapeutic drugs at continuous, lower doses, which generally result in fewer toxic side effects and reduced dependency on supportive therapies [8, 47, 48].

Among the drugs used in mCHT, Cyclophosphamide plays a significant role. It belongs to the group of nitrogenous mustard alkylating agents and primarily acts during the S phase of the cell cycle, exerting its effects by interfering with transcription and DNA replication processes [49, 50]. The DNA damage induced by Cyclophosphamide has been observed in both *in vitro* and *in vivo* studies, often assessed through the comet assay, a technique that detects DNA strand breaks [51, 52, 53, 54, 55, 56, 57, 58].

Living organisms are continually exposed to sources of DNA damage, both endogenous and exogenous, which can significantly impact health and contribute to various diseases [59]. Studies consistently show that patients with mamary cancer exhibit notably higher levels of initial endogenous DNA damage compared to healthy individuals [59, 60, 61]. In response to these genetic challenges, cells have evolved complex mechanisms to safeguard genome stability, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end-joining (NHEJ) [62, 63].

Based on the presented data, there is no apparent evidence of a substantial genotoxic effect associated with the administered treatment. Notably, despite a significant increase in DNA damage during the initial month of treatment, this damage markedly decreased during the second month and eventually returned to initial endogenous levels by the third month (FIG. 2). These findings suggest the possibility that patients developed increased tolerance to the low doses of Cyclophosphamide administered as the treatment regimen progressed. This enhanced tolerance may have facilitated the effective repair of DNA damage resulting from the underlying disease or the treatment itself.

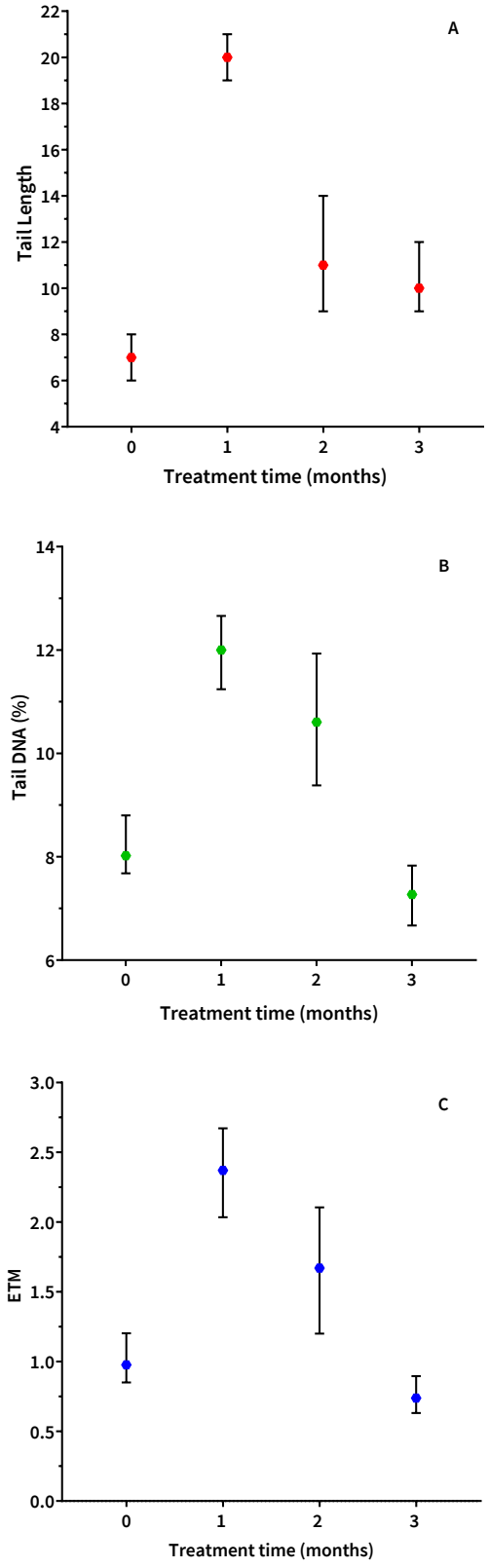


FIGURE 2. Medians along with their corresponding 95% confidence intervals for the parameters analyzed before and after three months of treatment in canine patients undergoing mCHT

The data presented in this study suggests that the population of canine patients under investigation, diagnosed with mammary cancer displayed evidence of effective DNA damage repair. Initially, these patients exhibited low levels of endogenous DNA damage. However, after one month of Cyclophosphamide treatment, there was a notable increase in DNA damage levels, likely a consequence of the treatment's mechanism of action.

The use of Cyclophosphamide in metronomic chemotherapy (mCHT) appears to offer a less aggressive alternative concerning its impact on blood parameters and genotoxic effects compared to conventional chemotherapy. This potential presents significant advantages for patients in terms of their overall well-being and their ability to tolerate the treatment. During the one-year clinical follow-up, significant outcomes were observed. Seven patients achieved a complete response to treatment, while two showed a partial response. Unfortunately, one patient was lost during this period, unrelated to cancer, succumbing to hemolysis induced by canine Ehrlichiosis.

The combined treatment approach, including mastectomy and mCHT, delivered notable benefits for canine patients with mammary carcinomas. Importantly, all patients survived and showed no neoplastic recurrence within the one-year evaluation. To establish the consistency and long-term sustainability of this trend among canine mammary cancer patients undergoing mCHT, further investigation with a larger sample size is needed to draw more comprehensive and statistically significant conclusions regarding treatment efficacy and durability in the context of mammary carcinoma in canines.

CONCLUSIONS

The study highlights the potential advantages of utilizing Cyclophosphamide in mCHT compared to conventional chemotherapy, as evidenced by its milder impact on blood parameters and genotoxic effects. Moreover, it suggests the effectiveness of DNA damage repair in patients undergoing mCHT. The clinical outcomes are encouraging, with seven patients achieving complete responses and two demonstrating partial responses to the treatment. Notably, all patients survived the one-year follow-up without any signs of neoplastic recurrence. However, it is imperative to emphasize the necessity for further research with a larger sample size to establish the long-term consistency and sustainability of these positive treatment results. This will allow for more comprehensive and statistically significant conclusions regarding the efficacy and durability of mCHT in the context of canine mammary carcinoma.

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Conflict of interests

The authors of this study declare that there is no conflict of interest with the publication of this manuscript.

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