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Original Research Article

Modulation of Cytokines by Ketogenic Diet and Cyclophosphamide Chemotherapy in 1-Methyl Nitrosourea-Induced Mammary Tumour in Rats

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ABSTRACT

Cancer is the second leading cause of death worldwide and it is expected to increase fivefold in the next 25 years. The influence of diet and nutrition in chronic diseases risk and prevention has been a subject of several studies for years. The aim of this study was to investigate the modulation of cytokine by cyclophosphamide and ketogenic diet on experimentally 1-Methyl Nitrosourea (MNU)-induced mammary tumour in female Wistar rats. Twenty-five female rats were divided into five groups of five rats each. Mammary tumour was induced with a single intraperitoneal administration of MNU (65 mg/kg). Blood samples were collected before and after treatment with ketogenic diet supplementation and co-administration of Cyclophosphamide (10 mg/kg) with ketogenic diet, serum concentrations of cytokines were measured. After the treatment, rats were euthanized, and the mammary glands were harvested for histological examination. Ketogenic diet supplementation and co-administration of Cyclophosphamide with ketogenic diet significantly ($P \leq 0.05$) reduced the serum concentrations of interleukin (IL)-6, 4 and 18, vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1). The treatments also significantly ($P \leq 0.05$) increased the serum concentrations of IL-2, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in MNU-induced mammary tumour. Histopathological examinations showed that the neoplastic growth in the treatment groups shifted to hyperplasia and benign carcinoma, rather than the severe adenocarcinoma detected in the untreated group. Ketogenic diet possesses a significant beneficial effect on cancer chemotherapy.

Keywords: Ketogenic diet, cytokines, MNU, Wistar rats.

Introduction

Cytokines and cytokine receptors play important roles in the development of malignant tumours as signaling molecules that recruit inflammatory cells into the tumour microenvironment.[1] They have an integral role in tumour induction and progression. They can assist the generation and conservation of tough antitumour immune reactions, but they can also contribute to chronic inflammation and promote tumour formation, growth and metastasis.[2]

Cancer is a term used to describe a large group of diseases in which abnormal cells divide without control and can affect any part of the body.[3,4] Breast cancer is the most common cancer and the second principal cause of cancer death in women worldwide.[5] It constitutes a major public health issue globally with over 1 million cases annually.[6] Breast cancer is therefore considered to be a major public health problem of great interest and importance to medical professionals in various specialties.[7]

Literature review has shown that researches have been reported on cancer management, but to date no drug has proven to be sufficiently effective. In addition, side effects have been a major concern in cancer treatment as a result an ideal drug for cancer treatment is currently not available.

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Ketogenic diet is any diet that causes ketone bodies to be produced by the liver, changing the body's metabolism from glucose and towards fat utilization. It is a diet that contains high fat, low carbohydrate, moderate protein combination.[8] Only few studies explore diet as a potential adjuvant to cancer treatment. Tumour tissue single out sugar for energy and often has problems burning fat for its energy demands. In contrast, the healthy cells of cancer patients often have difficulty in utilizing carbohydrates and the metabolism favours fat for energy.[3] Thus, a diet furnishing the cancer patient with sufficient fat and protein for his demands while limiting the carbohydrates tumours thrive on, could be a helpful strategy in improving the patients' situation. A ketogenic diet (KD) fulfills these requirements. The actions of this diet could be associated with modulation of cytokines.

The aim of this study was to investigate the modulation of cytokines by ketogenic diet supplementation on Cyclophosphamide chemotherapy in 1-Methyl Nitrosourea (MNU)-induced mammary tumour in female Wistar rats.

Materials and Methods

Materials

1-Methyl Nitrosourea (MNU) (Sigma-Aldrich, USA), Cyclophosphamide (Sigma-Aldrich, USA), olive oil (KSC, Tunisia), weighing balance, syringes and needles, distilled water, normal saline.

Animal management

Ethical approval number "DAC/IW-OT/408/14" was obtained from the Ahmadu Bello University committee on animal care (ABUCAC). Female wistar rats weighed (100-120 g) were obtained and housed in the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria. The animals were fed

with standard vital feed and given access to water *ad libitum* up to the end of the experiment.

Preparation of ketogenic diet
The nutritional profile of the diet was: fat 75%, protein 8%, carbohydrates, fiber, vitamins and minerals 17%. The diet was prepared according to the Bioserv F3666 ketogenic diet recipe and was modified by the replacement of cellulose with nut bran.[10] The diet was prepared from olive oil and groundnut, the olive oil was administered orally using an "oral gavage" starting with the dose of 10 mL/kg and triturated to a maximum of 100 mL/kg. Approximately ten milliliters of olive oil is equivalent to five gram, each gram of olive oil contains 9 kCal of energy, and therefore in every hundred milliliters there is approximately 450 kCal of energy. The olive oil serves as the main source of the fat (about 90%). About 1 kg of groundnut was shallow fried and ground in a grinding machine, 2 g of common salt was added to the groundnut and then the oil was extracted from the grinded groundnut using a mortar and pestle, after extracting the oil, the cake was shallow fried in a groundnut oil. About 20 g of the groundnut cake was subjected to proximate analysis. The groundnut cake was given as the normal diet. The groundnut cake contained the required amount of protein, carbohydrates, fiber, vitamins, minerals and the remaining fat.

Induction of cancer
Induction of cancer was carried out using the method of Thompson *et al.*[9] At 50 days of age, the rats were given a single dose (65 mg/kg) of 1-Methyl nitrosourea (MNU) dissolved in normal saline via the intraperitoneal route. The animals were observed and palpated weekly to determine the development, localization and size of neoplasias on the breast. Twenty-four weeks after tumour induction, blood samples were collected to evaluate the level of cytokines and chemokines concentrations to confirm the establishment of the tumour. The disease surviving animals after tumour induction were divided into four groups. Group one rats were treated with normal saline this serves as cancer control group. Group two rats were treated with ketogenic diet, group three rats were treated with Cyclophosphamide (10 mg/kg). Group four rats were treated with combination of ketogenic diet and Cyclophosphamide (10 mg/kg). The animals were weighed weekly; the treatment lasted for forty-two (42) days. Food intake by the animals was observed and weekly average food intake was recorded.

Collection of serum for cytokines assay
Optic nerve of each rat was punctured with a capillary tube, the nerve was squeezed with hand and blood was collected via the nerve. About 3 mL of blood was collected and placed in a 5 mL plain snap cap bottle. The blood was placed in a centrifuging machine and centrifuge under 40,000 RPM for twenty minutes, 0.25 mL of the serum was pipetted out from the centrifuged blood using a micro-pipette and placed in a 0.5 mL plane snap cap bottle. Equal volume of phosphate buffer (pH 7.4) was added to the serum,[10] the sample was packaged in a dry ice and taken to Eve Technologies in Canada for cytokine/chemokines multiplex assay (IL-2,4,6,18, VEGF, MCP-1, TNFα, and IFN-γ).

Multiplex analysis of cytokines
In this study we quantified 27 cytokine/chemokine biomarkers simultaneously by using a Discovery Assay® called the Rat Cytokine Array/Chemokine Array 27-Plex (Eve Technologies Corp, Calgary, AB, Canada). The multiplex assay was performed at Eve Technologies by using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and a Milliplex rat cytokine kit (Millipore, St. Charles, MO, USA). The 27-Plex consisted of EGF, Eotaxin, Fractalkine, G-CSF, GM-CSF, GRO/KC/CINC-1, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, Leptin, LIX, MCP-1, MIP-1α, MIP-2, RANTES, TNFα and VEGF. The assay sensitivities of these markers range from 0.1- 15.7 pg/ml. Individual analyte values and other assay details are available on Eve Technologies' website or in the Milliplex protocol.

Statistical analysis
Statistical analysis was carried out using Statistical Package for Social Scientist (Version 20) and data obtained were expressed as mean ± SEM. Difference between mean was analyzed using spilt plot analysis of variance (ANOVA) and followed by Bonferoni post hoc test, for multiple comparison, values with $p < 0.05$ were considered significant.

Results and Discussion

Interleukin-2, 4, 6, and 18, VEGF, MCP-1, TNFα, and IFN-γ were found to be significantly ($p < 0.05$) higher in the sera of rats induced with cancer than in the sera of normal control before commencement of treatment. Post treatment, the serum concentrations of MCP-1, IL-4,6,18, and VEGF in the animals treated with Cyclophosphamide, were significantly ($p < 0.01$) reduced compared to control. Similarly, animals treated with Cyclophosphamide/ketogenic diet supplementations significantly ($p < 0.05$) reduced when compared to the untreated group. So also, in the cancer control group, the serum concentration of VEGF increases significantly ($p < 0.01$). Treatment with Cyclophosphamide significantly ($p < 0.05$) increased the serum concentrations of IL-2, TNFα, and IFN-γ. Similarly, animals treated with Cyclophosphamide/ketogenic diet supplementation significantly ($p < 0.01$) increased the serum concentration of TNFα when compare to the cancer control group. In the cancer control group, the serum concentration of IL-2 and TNFα significantly ($P < 0.05$) decrease (Figures 1-4).
The photomicrograph section of the mammary gland of female Wistar rat showed normal lactiferous glands, while the section of the mammary gland of female Wistar rat induced with MNU showed severe intraductal carcinoma of the lactiferous glands. While the sections of the mammary gland of female Wistar rat induced with MNU and treated with Cyclophosphamide, Cyclophosphamide co-administered with ketogenic diet, and ketogenic diet alone, showed fibrosis with high mononuclear cellular infiltration, hyperplasia and local invasiveness of the lactiferous glands (Plate 1).
There was significant increase in the serum concentration of MCP-1, VEGF, TNF-α, IFN-γ, IL-2, IL-6, IL-4, and IL-18 in the sera of the MNU-induced rats than the normal rats, these results are thus in agreement with previous studies conducted by Seruga *et al.* (2008)[11] which shows that, increased levels of circulating cytokines and their receptors were found in observational studies of patients with various types of cancers. Treatment with Cyclophosphamide and Cyclophosphamide co-administered with ketogenic diet supplementation significantly decrease the level of; MCP-1, VEGF, IL-6, IL-4, and, IL-18 and also significantly increase TNF-**α, IFN-γ** and IL-2, in the sera of MNU-induced mammary tumour in rats.
Reduction in concentration of MCP-1, IL-18, IL-4, IL-6, and VEGF could be associated with the immunosuppressive effect of Cyclophosphamide and also the antitumour activity of Cyclophosphamide and ketogenic diet. The decrease of MCP-1 observed in the sera of rats post treatment with Cyclophosphamide, and Cyclophosphamide co-administered with ketogenic diet may be as a result of their antitumour activity. MCP-1 was reported to play crucial roles in neovascularization and progression of disease in patients with breast carcinoma, its expression was also reported to be a phenotype of tumour aggressiveness in various types of tumours, including carcinoma of the breast.[12-14]
Tumour microenvironment is rich in cytokines and other inflammatory mediators that influence immune suppression, growth of cancer cells, tissue remodeling and angiogenesis.[15] These cytokines are interrelated and may act in an additive manner, suggesting that these cytokines form a network of interrelated factors that may affect tumour cell progression in a cooperative manner. There is a connection between the degree of cytokines/chemokines expression with cancer aggressiveness and prognosis in patients with breast cancer.[16]
The decrease of VEGF observed in the sera of treated rats may be as a result of their antitumour activity. High VEGF concentrations in the blood have also been found in patients with cancer of the breast.[16] There has been a series of reports of connections between the degree of VEGF expression with tumour aggression and prognosis in patients with cancer of the breast.[16-18] VEGF was found to possess proangiogenic effects and stimulate angiogenesis that is essential for supporting the growth of tumours it has also been reported to participate in the pathogenesis of neoplastic disease in many studies.[16, 19,20] This is also in line with the results obtained from this study; the untreated group has the highest VEGF concentration, and thus the worst survival time. The group with the least VEGF concentration showed a better prognosis and prolonged survival time. Hence, decreased level of VEGF in the rat's sera after treatment with Cyclophosphamide co-administered with ketogenic diet supplementation may be a good treatment in breast cancer.

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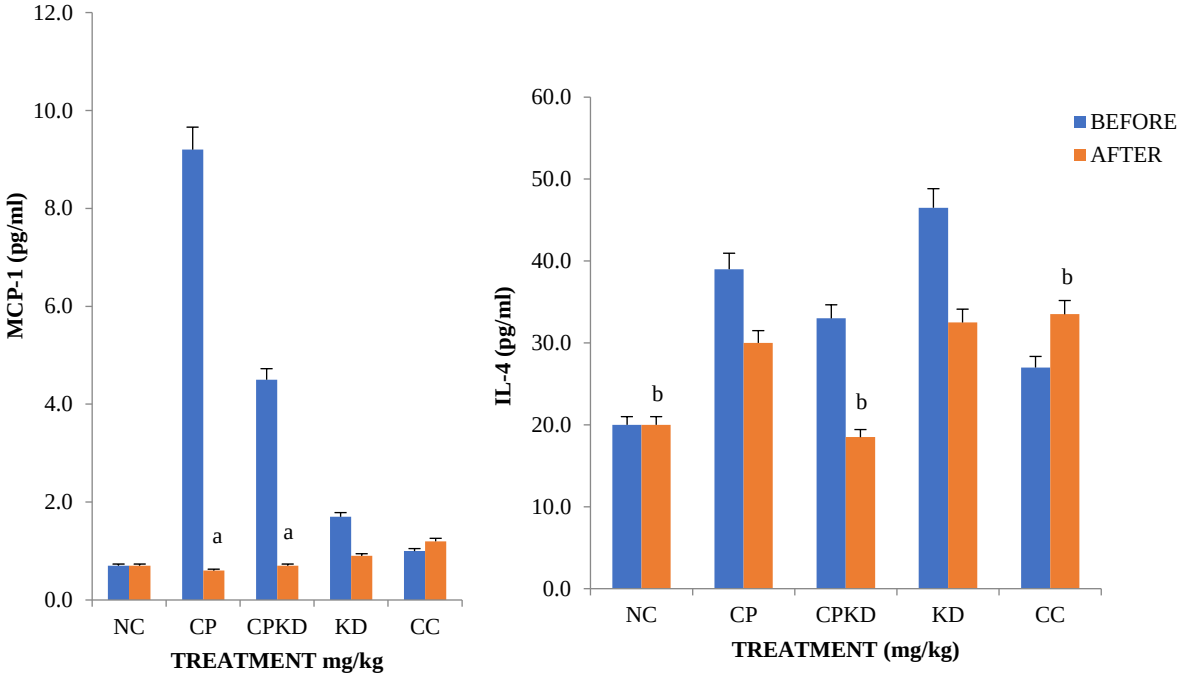

Figure 1: Serum concentrations of MCP-1 and IL-4 before and after treatment with ketogenic diet, Cyclophosphamide, and Cyclophosphamide co-administered with ketogenic diet.
Data was analyzed using split plot ANOVA, followed by Bonferoni post hoc test Significant at P =0.001 NC = Normal Control, KD = ketogenic diet, CP = Cyclophosphamide, CPKD = Cyclophosphamide and ketogenic diet, CC = Cancer control. a = significant within the group, b = significant between the groups, n = 4.

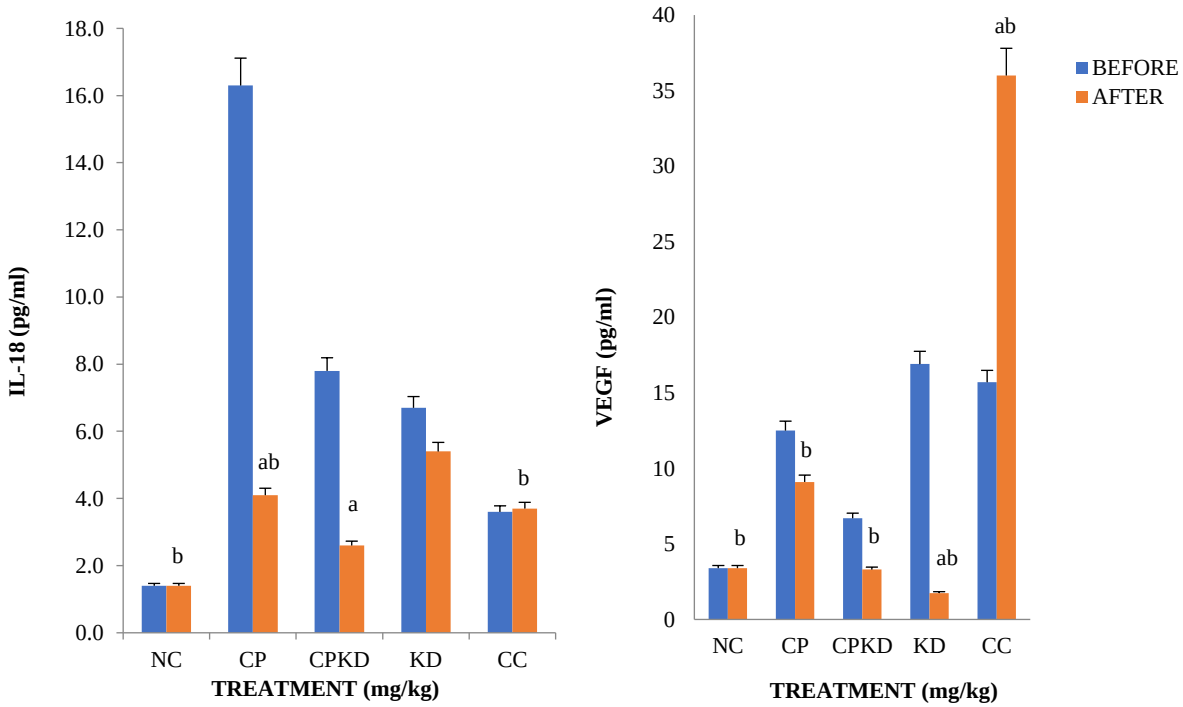

Figure 2: Serum concentrations of IL-18 and VEGF before and after treatment with ketogenic diet, Cyclophosphamide, and Cyclophosphamide co-administered with ketogenic diet.
Data was analyzed using split plot ANOVA, followed by Bonferoni post hoc test significant at P =0.005NC = Normal Control, KD = ketogenic diet, CP = Cyclophosphamide, CPKD = Cyclophosphamide and ketogenic diet, CC = Cancer control. a = significant within the group, b = significant between the groups, n = 4.

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The increase in the concentration of TNF-α, IFN-γ and IL-2 found in the sera of MNU-induced mammary tumour rats was due to the stimulation of inflammatory mediators associated with cancer cells. The further increase in the serum concentrations of TNF-α, IFN-γ and IL-2 observed after treatment could be due to their antitumour activities observed in the study. TNF-α is a multifunctional cytokine involved in apoptosis, inflammation, as well as immunity,[21] thus, it is suggested that increased level of TNF-α was possibly linked to the activation of NF-κB,[22] which plays a crucial role in inflammation and carcinogenesis.[23] These results are thus in agreement with previous studies conducted by Seruga *et al.*[11] which shows that, in an animal model, treatment with aromatase inhibitor in breast cancer increases the production of pro-inflammatory cytokines such as TNF-α. It was also reported that, increase in IL-2 concentration stimulates the release of other cytokines such as TNF-α and IFN-γ. One of the possible mechanisms of antitumour of Cyclophosphamide and ketogenic diet could be activation of this cytokine (TNF-α).

IFN-γ was reported to have paradoxical effects on tumour cells. Zhou *et al.*[19] reported that IFN-γ may slow proliferation of some gastric cancer cells by affecting the cell cycle to play a negative role in the development of gastric cancer. It was also reported to be an important inflammatory factor involved in immune regulation, and has direct and indirect anti-tumour effects,[23, 24] as well as anti-viral effects. IFN-γ may directly or indirectly affect apoptosis of some cancer cells, suppress angiogenesis, inhibit proliferation and also induce differentiation of cells.[23] The increased concentrations of IFN-γ observed may be due to the effects of Cyclophosphamide and ketogenic diet which could probably stimulate IFN-γ and hence could be one of the mechanisms of their antitumour activities.

IL-2 induces antitumour activity by increasing numbers of NK and MHC-restricted tumour-specific cytotoxic cells.[11] This finding is in line with the study conducted by Gorelik, [26]who reported that IL-2 activates killer T-cells, natural killer cells, cell mediated tumour cell death and also signal the release of secondary cytokines (IFN and TNF-α). Thus, the effect observed in this study might have probably acted via the same way as above.

Jiang *et al.*[27] also reported that, IL-6 plays an important role in maintaining the growth of MCF-7 breast cancer cells and also suggested that careful modulation of IL-6 and IL-6R expression of cells as a potential approach for breast cancer therapy. It also plays an important role in the neoplastic process through it action on cancer cell-adhesion, motility, proliferation, tumour-specific antigen expression and thrombopoiesis.[26] IL-6 mediates its effects via binding to cell surface receptors, IL-6R, which are active in both membrane-bound and soluble forms.[26]

The decrease in concentration of IL-6 observed in the sera of the rats treated with cyclophosphamide, and cyclophosphamide co-administered with ketogenic diet could be associated with the antitumour activities observed. Based on the data obtained from this study, Cyclophosphamide and ketogenic diet supplementation may exert their antitumour activity via down regulation of the expression of IL-6.

Ketogenic diet supplementation and co administration of Cyclophosphamide and ketogenic diet supplementation significantly reduced the serum concentrations of interleukin-6, 4 and 18, VEGF and MCP-1 and also significantly increased the serum concentrations of IL-2, TNFα and IFNγ in MNU-induced mammary tumour in Wistar rats. Reduction in the serum concentration of these cytokines and chemokines denotes a good prognosis and inhibition of cell proliferation and also metastases. Based on the result obtained, ketogenic diet may act by inhibiting the secretion of MCP-1, IL-4, IL-6, IL-18, and VEGF, and also stimulating the secretion of IL-2, IFN-γ and TNF-α to exert its antitumour activity. Ketogenic diet in combination with Cyclophosphamide may be a better approach in the treatment of breast cancer. This result is in agreement with previous studies conducted. Diet remedies like Ketogenic diet have been found to be effective in the management of cancer. Researchers have shown that Ketogenic diet seems to have the potential to manage advanced cancer cases.[27-30]

In summary we found that the sera of rats induced with mammary tumour express higher levels of multiple cytokines than the sera of normal rats, and also treatments with Cyclophosphamide and ketogenic diet positively modulate the expression of MCP-1, IL-18, IL-4, IL-6, and VEGF, IL-2, TNF-α and IFN-γ in the sera of MNU-induced mammary tumour rats.

Histopathological examinations showed that the neoplastic growth in the treatment groups shifted to hyperplasia and benign tumours, rather than the severe adenocarcinoma detected in the untreated group.

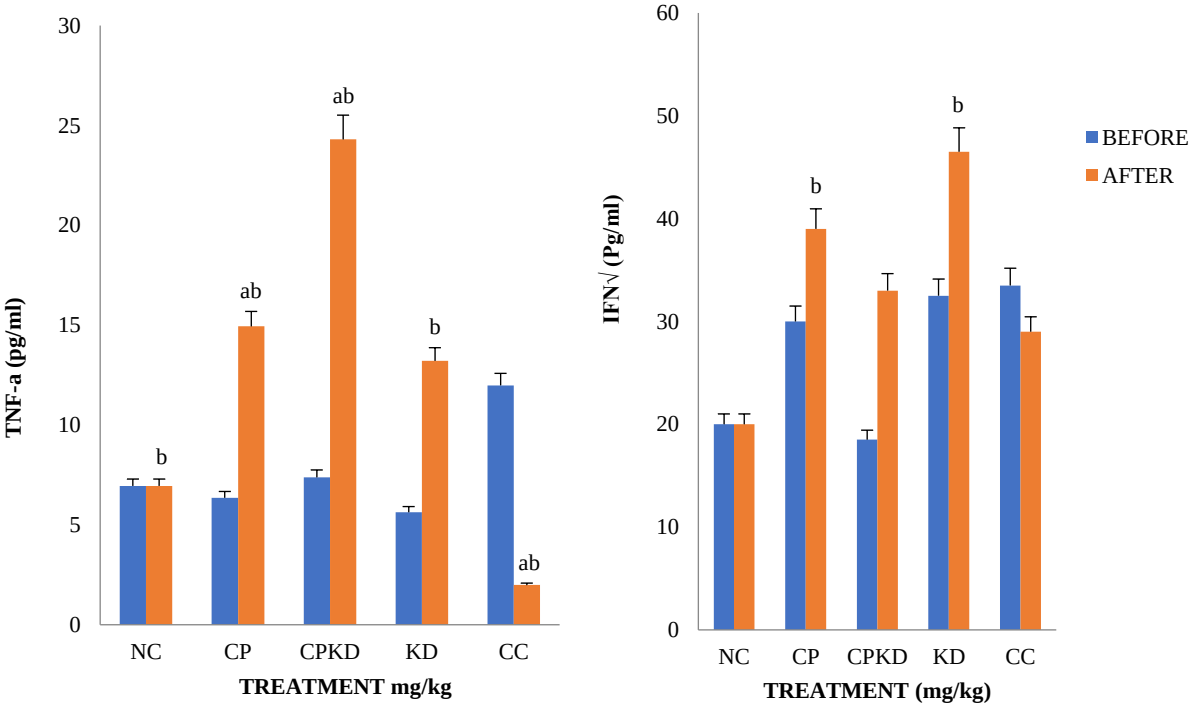

Figure 3: Serum concentrations of TNF-α and IFNγ before and after treatment with ketogenic diet, Cyclophosphamide, and Cyclophosphamide co-administered with ketogenic diet.

Data was analyzed using split plot ANOVA, followed by Bonferoni post hoc test significant at $P \leq 0.05$ NC = Normal Control, KD = ketogenic diet, CP = Cyclophosphamide, CPKD = Cyclophosphamide and ketogenic diet, CC = Cancer control. a = significant within the group, b = significant between the groups, n = 4.

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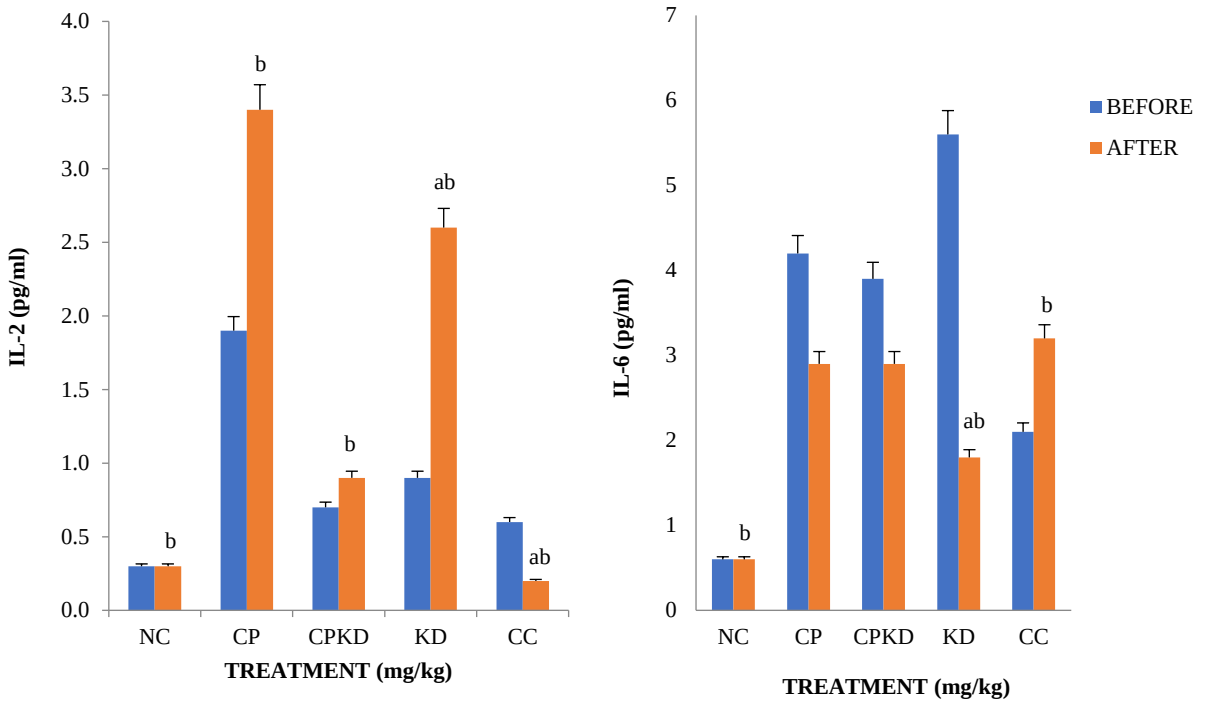

Figure 4: Serum concentrations of IL-2 and IL-6 before and after treatment with Cyclophosphamide, and Cyclophosphamide co-administered with ketogenic diet.
Data was analyzed using split plot ANOVA, followed by Bonferoni post hoc test significant at P =0.001, NC = Normal Control, KD = ketogenic diet, CP = Cyclophosphamide, CPKD = Cyclophosphamide and ketogenic diet, CC = Cancer control. a = significant within the group, b = significant between the groups, n = 4.

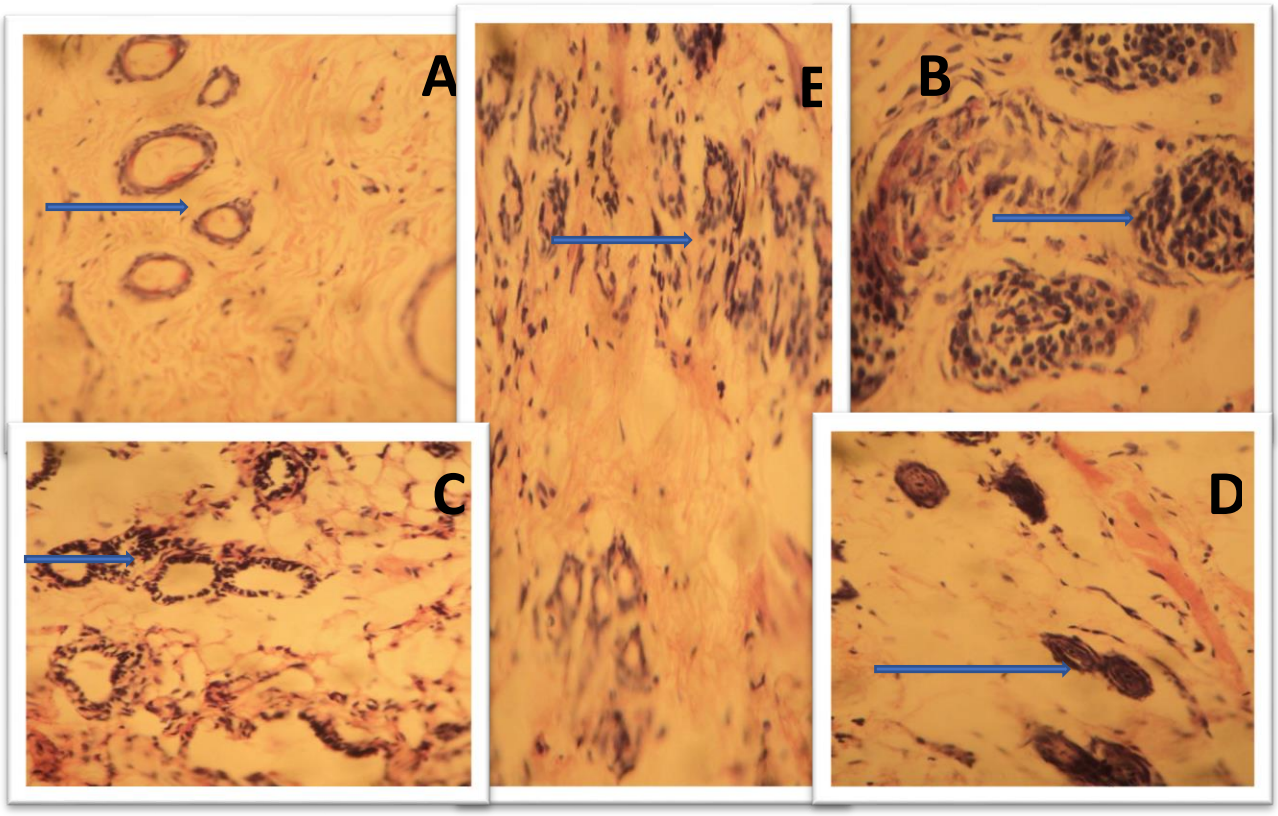

Plate 1: Photomicrograph of the mammary gland of female Wistar rats showing lactiferous gland (blue arrow) (H and E X 400); A = normal control, B = cancer control, C = ketogenic diet, D = Cyclophosphamide, E = Cyclophosphamide co-administered with ketogenic diet.

Conclusion

Ketogenic diet supplementation with Cyclophosphamide chemotherapy was found to positively modify cytokines concentrations in 1-Methyl Nitrosourea-induced mammary tumour.

Conflict of interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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