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Impact of vitamin B_{12} and folic acid co-treatment on MCF-7 cells

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Abstract
This study investigated the impact of vitamin B_{12} and folic acid (also known as vitamin B_9 or folate) on MCF-7, a human breast cancer cell line, for 24, 48, and 72 hours. This research sought to elucidate the potential role of these methyl-group-contributing nutrients in modulating cancer cell growth to offer insights that can be utilized in the field of cancer therapy. The results consistently revealed that, at all-time points, there were no statistically significant differences in cell proliferation and viability, between the treatment and control groups. These findings strongly indicated that the introduction of vitamin B_{12} and folic acid to MCF-7 cell cultures did not trigger changes in cell functionality in terms of proliferation and viability. The collective outcomes underscore that the administration of vitamin B_{12} and folic acid, as outlined in this study, is unlikely to induce noteworthy alterations in the examined cellular parameters of cancer cells. These results affirm the safety and potential suitability of these vitamins as therapeutic nutrients for future applications.

Keywords: Vitamin B_{12}, Folic acid, MCF-7, Cancer therapy, Cell proliferation, Cell viability

Introduction
Some of the micronutrients associated with cancer are those involved in one carbon cycle, including folic acid, vitamin B_{12}, choline, and methionine. These nutrients are crucial for human health, serving as essential suppliers of methyl groups involved in transmethylation reactions. They play pivotal roles in DNA synthesis and methylation, nucleoprotein and cell membrane formation, phospholipid processing, and transmembrane signal transduction. These processes collectively contribute to vital cellular functions such as cell proliferation, metabolism, viability, and genetic integrity (Institute of Medicine, 1998) [6]. These mechanisms are essential for normal cell development and division, and disturbances in these processes may result in cancer. However, the link between vitamin B12, folic acid, and cancer is complicated and encompasses multiple mechanisms.

Breast cancer is the most frequently diagnosed neoplastic condition, asserting its dominance as the leading cause of cancer-related deaths among women worldwide. This disease accounts for a substantial 23% of all cancer cases and claims the lives of 14% of those afflicted by malignancies in this category. Notably, nearly half of all breast cancer cases and a significant 60% of its fatal outcomes are projected to occur in economically developing regions, as reported by Jemal *et al.* (2011) [7].

Patients diagnosed with cancer have been documented to exhibit increased plasma levels of vitamin B_{12}, which had raised concerns about the safety of vitamin B_{12} supplementation. In a study by Ebbing *et al.* (2009) [4] in Norway, the combination therapy of folic acid and vitamin B_{12} was associated with increased cancer-related outcomes and overall mortality in individuals with ischemic heart disease. However, there exists an insufficiency of empirical support to posit a causal relationship between elevated plasma vitamin B_{12} concentrations, high dietary intake of B_{12}, or the administration of pharmacological doses of vitamin B_{12} and folic acid and the development of cancer. Later studies by Singh *et al.* (2019) [15], showed supplementation with Vitamin B_{12} and folic acid, mitigates hematologic toxicity induced by pemetrexed-based chemotherapy. They stated that commencing vitamin B_{12} and folic acid simultaneously with a pemetrexed-platinum doublet chemotherapy regimen did not result in amplified hematologic toxicity. Obeid, (2022) [12] also suggested that it is imperative to diagnose and manage low vitamin B_{12} status in cancer patients to avert the haematological and neurological complications stemming from deficiency.

However, the association between vitamin B_{12} and folic acid supplementation and cancer risk is still being studied, as excessive consumption of these supplements may encourage the growth of existing cancer cells in some circumstances. Therefore, further studies are warranted to ascertain the safety of these micronutrients in cancer therapy and their effect on cancer cells.

For our experiment, we utilized the Michigan Cancer Foundation (MCF) cell line, which was established from pleural effusions of advanced breast adenocarcinoma metastases at the Michigan Cancer Foundation in 1973. This cell line holds the distinction of being the most extensively studied human breast cancer cell line in the world (Lee *et al*., 2015) [9]. The aim of our study was to investigate the effects of vitamin B_{12} and folic acid on the proliferation and viability of the human breast cancer cell line MCF-7 over 24, 48, and 72 hours to explore the interplay of this methyl-group containing nutrients in cancer biology and their implications in breast cancer therapeutic approaches.

Materials and Methods

The human breast cancer, Michigan Cancer Foundation-7 (MCF-7) cell line procured from National Centre for Cell Science; Pune, Maharashtra was used for this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 per cent foetal bovine serum, one per cent antibiotic antimycotic solution (10,000 U Penicillin, 10mg Streptomycin, and 25µg Amphotericin B per mL in 0.9 per cent normal saline). The cells were plated in 24-well plates at a plating density of 0.05×10^6 cells per well for proliferation studies and 0.01 x 10^6 cells per well in 96 well plate for viability studies. The cells were incubated with control and treatment media (Table 1) for 24h, 48h and 72h in CO_2 incubator at 37 °C with 95 per cent oxygen and five percent carbon dioxide.

Table 1: Media composition

Groups	Treatments		
	TCDD	**Vitamin B_{12} (5000pg/mL)**	**Folic acid (50ng/mL)**
Control	-	-	-
T1	-	Vitamin B_{12}	Folic acid

Cell proliferation studies

Treatment media were prepared as shown in Table 1. After 24 h of seeding (Fig. 1), cell growth media from plates were discarded; control and treatment media were added to appropriate wells and were kept for 24 h, 48 h, and 72 h respectively in a CO_2 incubator. The treatment media was changed every 24 hours until the completion of the experiment.

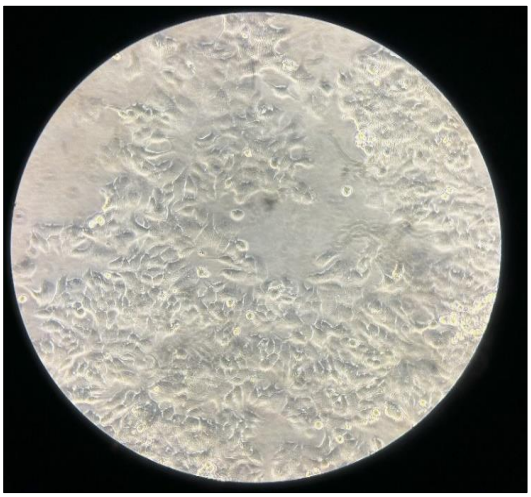

Fig 1: MCF-7 cell (20X)

After 24, 48, and 72 h of treatment, cells were trypsinised, and the cell pellet was collected and dispersed in 1mL of sterile PBS. The cell proliferation was estimated by the trypan blue dye exclusion method using haemocytometer. One part of cell suspension (10µl) was mixed with one part of 0.4% Trypan blue (10µL) and incubated for 3 minutes at room temperature. Trypan blue/cell mixture was applied to a haemocytometer chamber and the viable cells were counted in each of the four corner squares of the haemocytometer. The following formula was applied to calculate the number of cells from one well that was suspended in 1mL PBS.

Total number of cells/mL = average cell counts per square x dilution factor x 10^4

Cell viability studies

The impact of vitamin B_{12} and folic acid on viability of MCF-7 cells was evaluated using the 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) reduction assay, measuring mitochondrial metabolic activity. Cell suspension was added into the wells of 96 well plates to have 0.01 x 10^6 cells per well. After 24h of seeding the cells were incubated with control and treatment media (Table 1) for 24, 48, and 72 hours. The media were changed every 24 hours and fresh media was added to each well.

After 24 h, 48h, and 72h of incubation the media were carefully pipetted out. 200 µL of fresh media without FBS was added to all the wells including blanks. Ten microliters of MTT (five mg/mL prepared in DPBS) were added to all wells including blanks. The plates were incubated in dark at 37°C for 4 hours, in a CO_2 incubator. After incubation, the media containing MTT was removed and 200 µL of DMSO was added to all wells including the blanks. The plates were gently agitated on an orbital shaker for 10 m. The absorbance was measured using a microplate reader (Multiskan Skyhigh Tc Mdrop) at a wavelength of 570 nm.

The percent cell viability was calculated using the formula below.

Percent cell viability= (Average absorbance of treated cells/Average absorbance of untreated cells) x 100

Results

Cell proliferation studies

Following treatment with vitamin B_{12} (5000 pg/mL) and folic acid (50 ng/mL), MCF-7 cell proliferation was analysed at 24, 48, and 72-hour intervals, with results compared to a control group. There were no significant differences in cell count, and microscopic examination revealed no morphological changes. Cell proliferation in the control group treated with vitamin B_{12} and folic acid remained similar to the untreated control group at all time points. These findings suggest that this particular treatment regimen did not impact MCF-7 cell proliferation (Table2; Fig. 2).

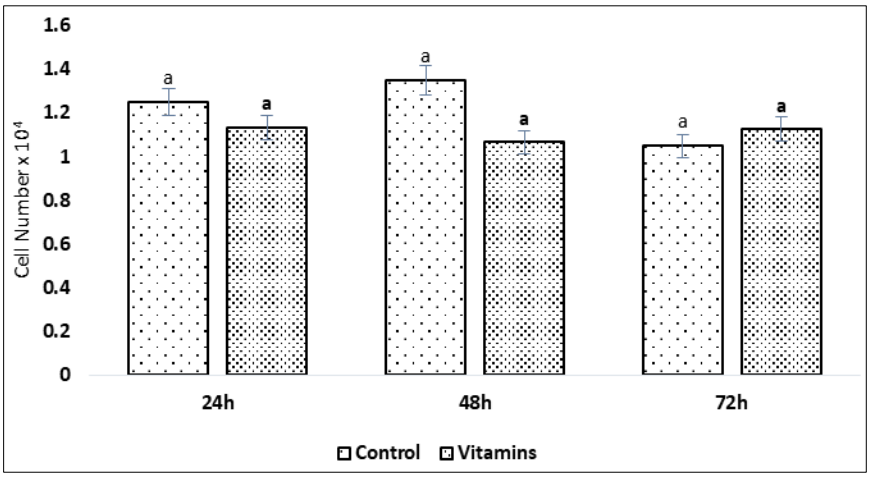

Fig 2: Effect of vitamin B_{12} and folic acid on cell proliferation

Table 2: Different superscripts denote significant difference between values; *($p \leq 0.05$) difference from control value

Time	C (Cell No. x 10^4)	CV (Cell No. x 10^4)
24h	1.25±0.18[a]	1.13±0.25[a]
48h	1.35±0.25[a]	1.06±0.18[a]
72h	1.05±0.09[a]	1.13±0.10[a]

Cell viability studies

The investigation focused on the effects of vitamin B_{12} (5000 pg/mL) and folic acid (50 ng/mL) on MCF-7 cell viability over 24, 48, and 72 hours, compared to a control group. At each time point, treated cells displayed no significant difference in viability compared to the control. There were consistent findings over the entire 72-hour experimental period, indicating that the addition of vitamin B_{12} and folic acid did not cause notable changes in cell viability (Table 3; Fig. 3). These results emphasized the stability of cell viability under the specified treatment conditions and time intervals.

Table 3: Different superscripts denote significant difference between values; *($p \leq 0.05$) difference from the control value

Time	C (viability %)	CV (viability %)
24h	100[a]	93.9±6.19[a]
48h	100[a]	93.98±10.08[a]
72h	100[a]	100.43±5.14[a]

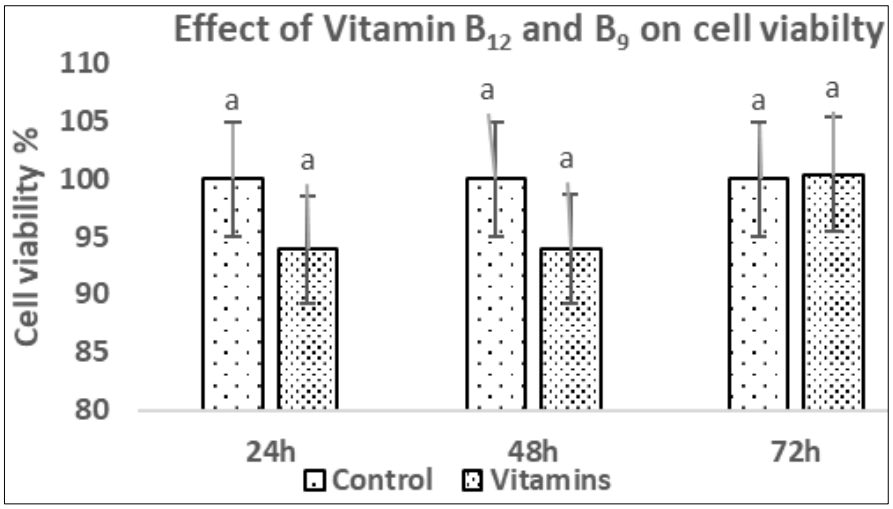

Fig 3: Effect of vitamin B_{12} and folic acid on cell viability

Discussion

Both vitamin B_{12} and folic acid are vital for overall health, and they are essential micronutrients that play crucial roles in various bodily functions. Experts have emphasized the protective influence of folic acid and folate against neural tube defects and maintaining adequate folate levels not only

diminishes the risk of cancer development but also mitigates the likelihood of experiencing strokes and neurodegenerative disorders. While a diet abundant in natural folate-rich foods is advantageous for overall health, intake of necessary daily folate levels is necessary for the prevention of neural tube defects (Berry *et al*., 2010) [1]. US Preventive Services Task Force endorses the continued practice of supplementing with 400–800 µg of folic acid (FA) per day for women intending to become pregnant (US Prev. Serv. Task Force. 2017). Vitamin B_{12} is a crucial micronutrient that functions directly as a coenzyme in the metabolism of folate. Reduced B_{12} levels exhibit a correlation with decreased concentrations of serum or red blood cell (RBC) folate (Chen *et al*., 2019: Nasari *et al*., 2015) [2, 11]. A deficiency of maternal vitamin B_{12} heightens the vulnerability of offspring to neural tube defects (NTDs) (Molloy, 2018; Nasri *et al*., 2015; Peker *et al*., 2016; Senousy *et al*., 2018) [10, 11, 13, 14]. Furthermore, an independent connection has been established between diminished maternal vitamin B_{12} levels and the risk of neural tube defects (NTD), irrespective of folate levels (Peker *et al*., 2016) [13].

Some studies have explored the use of folic acid and vitamin B_{12} in combination with chemotherapy drugs to mitigate the side effects without compromising the effectiveness of treatment. Though there was a rise in the detection of colorectal cancer cases in the United States and Canada in 1996, due to the introduction of folic acid fortification, a subsequent decrease in both the incidence of colorectal cancer and associated mortality has been noted from 1998 onwards. This decrease coincided with the introduction of mandatory folic acid fortification (Keum and Giovannucci, 2014) [8]. Information extracted from the Australian Cancer Database indicated a declining trend in colorectal cancer, significantly more prominent in 2010, aligning with the implementation of mandatory folic acid fortification (Vander Pols *et al*., 2021) [17]. Folic acid fortification was linked to a substantial 62% reduction in the occurrence of neuroblastoma. Specifically, this reduction translated to a decline from 1.57 cases per 10,000 children prior to fortification to 0.62 cases per 10,000 children following the fortification initiative (French *et al*., 2003) [5]. According to, Yang *et al*. (2013) [18], high dosages of vitamin B_9 and vitamin B_{12} supplementation reduced pemetrexed cytotoxicity, and supplementation with these vitamins resulted in higher rates of survival in pemetrexed-treated patients. Supplementation with vitamin B_{12} and folic acid decreased hematopoietic toxicity in pemetrexed-based chemotherapy (Singh *et al*., 2019) [15].

However, the relationship between vitamin B_{12}, folic acid, and cancer is complex, and research in this area continues to evolve. According to Ebbing *et al*. (2009) [4] in Norway, where there is no folic acid fortification of foods, treatment with folic acid plus vitamin B12 was associated with increased cancer outcomes and all-cause mortality in patients with ischemic heart disease. Yang *et al*. (2013) [18] postulated that a deficiency in dietary folate might potentially contribute to the early phases of cancer initiation, while substantial folic acid intake through supplements could potentially augment the proliferation of cancer cells. Patients with cancer have been observed to have higher plasma concentrations of vitamin B_{12}, raising concerns about the vitamin's safety (Obeid, 2022) [12]. A lack of folate in the diet may increase the early stages of cancer formation, but large quantities of folic acid in supplements may promote cancer cell growth (https://www.nutri-facts.org/en_US/news/articles/vitamin-b9--folic-acid--and-vitamin-b12-supplements-linked-with-.html).

In the present study, the effect of vitamin B_{12} (5000 pg/mL) and folic acid (50 ng/mL), on MCF-7 cell proliferation was analysed at 24, 48, and 72-hour intervals, with results compared to a control group. There were no significant no morphological changes in the cells due to treatment with these vitamins. Treated cells consistently exhibited proliferation rates and viabilities that closely paralleled those of the untreated control group across all monitored time intervals thus demonstrating that the particular treatment regimen in question did not exert any discernible influence on the proliferation and viability of MCF-7 cells. These results underscore the stability of MCF-7 cell viability under the specified treatment conditions and time intervals. Further investigation is warranted to explore the metabolic and genetic factors influencing whether supplementation with vitamin B_{12} and folic acid may impart any adverse effects at the cellular level. It is also imperative to conduct additional prospective investigations to establish the most effective nutrient remediation levels and future assessments of cancer risk may also encompass an examination of gene-nutrient interactions and their interplay with environmental factors (Ebbing *et al*., 2009; Drake and Colditz, 2009) [4, 3].

Conclusion

This study examined the influence of vitamin B_{12} and folic acid on the MCF-7 breast cancer cell line over 24, 48, and 72 hours. The investigation revealed consistent and statistically non-significant differences in cell proliferation and viability between the treatment and control groups at all time points. In summary, our investigation into the impact of Vitamin B_{12} and folic acid on the MCF-7 cancer cell line has yielded crucial insights. Despite concerns surrounding the potential for these vitamins to exacerbate cancer progression, our comprehensive study has conclusively demonstrated their lack of effect on both cell proliferation and viability within this specific context. This finding underscores the safety profile of vitamin B12 and folic acid concerning their direct influence on MCF-7 cells, suggesting that their utilization in addressing chemotherapy-related complications remains viable without concerns of inadvertently supporting cancer cell growth or survival. As such, these vitamins exhibit promise as adjunctive therapies, supporting their continued consideration in the management of chemotherapy-related complications without posing a direct risk of fostering cancer progression in this cellular model. Nevertheless, further investigations exploring these vitamins in different dose levels in diverse cellular contexts and *in vivo* models are warranted to comprehensively delineate their role and safety implications in cancer therapy. Such studies could provide invaluable insights into refining therapeutic strategies and optimizing the use of these essential vitamins in cancer treatment protocols

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