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Table of contents

List of Figures

List of abbreviations and definitions

A2780	Ovarian carcinoma
A431	Epidermoid carcinoma
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
CaCo-2	Colon adenocarcinoma
cis, trans	Specific positional isomer, having either a cis, trans or trans, cis configuration
CLA	Conjugated linoleic acid
CLA-PN	Conjugated linoleic acid – PharmaNutrients preparation
CLA-MAT	Conjugated linoleic acid – Matreya preparation
C-myc	A regulator gene involved in cancer cell proliferation
COX-2	Cyclooxygenase-2, an enzyme involved in the conversion of arachidonic acid to prostaglandin H2
CRT	Chemoradiotherapy
DLD-1	Colon adenocarcinoma
EGFR	Epidermal growth factor receptor
ErbB2	Erythroblastic oncogene B2, a human gene
ErbB3	Erythroblastic oncogene B3, a human gene
Fas	A trans-membrane protein, involved in the progression of cancer
Hep-G2	Hepatocyte carcinoma
HT-29	Colon adenocarcinoma
Ki67	Antigen Ki-67, a cellular marker for proliferation

LA	Linoleic acid
MCF-7	Breast adenocarcinoma
MIP-101	Colon adenocarcinoma
MP-2	Matrix metalloproteinase-2
mM	Millimolar
PC-3	Prostate adenocarcinoma
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma, a transcription factor regulating adipogenesis, lipid metabolism, cell proliferation and inflammation
SGC-7901	Gastric carcinoma
SKOV-3	Ovarian adenocarcinoma
TNF-α	Tumour necrosis factor-α, a cell signalling protein involved in inflammation
µM	Micromolar
5-LOX	Arachidonate 5-lipoxygenase, involved in arachidonic acid metabolism

The Effects of Conjugated Linoleic Acid on Human Health

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Abstract

Conjugated linoleic acid (CLA) is a family of 28 positional and geometrical isomers of linoleic acid (LA), found predominantly in the meat of ruminant animals. The health benefits of CLA have been widely researched, with specific interest into its anti-obesity and anti-carcinogenic properties. Conclusions from *in-vivo* studies have suggested that, with further research, CLA supplementation may be used in conjunction with current treatments for breast cancer and rectal cancer. *In-vitro* research into the anti-carcinogenic effects of CLA has revealed that different CLA isomers affect cancer cells through several different pathways. The anti-proliferative effects of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA have been demonstrated *in-vitro*, specifically on colon cancer, breast cancer, and prostate cancer. Ultimately, it has been concluded that the anti-proliferative effects of CLA isomers are dependent upon the type and malignancy of the cancer cells targeted. After reviewing the literature, it is clear that there is a gap in the research. To our knowledge, no study has ever tested the effects of CLA on the proliferation of epidermoid carcinoma cells, specifically the *cis*-9, *trans*-11 CLA isomer. This research could add to the growing body of evidence surrounding the effects of specific CLA isomers on different types of cancer *in-vitro*.

Conjugated linoleic acid (CLA) is a collective term for 28 positional and geometrical isomers of linoleic acid (LA), found primarily in the meat of ruminant animals (Koba & Yanagita, 2014). CLA is an 18-carbon polyunsaturated fatty acid, with double bonds at positions 8 and 10, 9 and 11, 10 and 12 or 11 and 13, each occurring in a *cis*-*trans*, *trans*-*cis*, *cis*-*cis*, or *trans*-*trans* configuration (Evans, Brown & McIntosh, 2002) (see Figure 1). The double bonds of CLA are separated by a single bond, providing the name 'conjugated' (Evans et al., 2002). CLA is synthesised in the gut of ruminant animals through the incomplete bio-hydrogenation of LA (Silva, 2014). Naturally occurring CLA predominantly consists of the *cis*-9, *trans*-11 isomer, also known as rumenic acid (Kramer et al., 1998). The principle dietary sources of CLA are beef and lamb, and ruminant dairy products, such as milk, butter and yoghurt (Chin, Liu, Storkson, Ha, & Pariza, 1992). Over the past three decades, CLA has gained significant scientific attention, after its anti-cancer (Ha, Grimm, & Pariza, 1987) and anti-obesity (Park et al., 1997) properties were discovered (Kim, Kim, Kim, & Park, 2016). According to Koba and Yanagita (2014) and Kim et al. (2016), recent reviews suggest that the physiological effects of CLA depend on its constituent isomers, with the *trans*-10, *cis*-12 isomer showing anti-carcinogenic, anti-obesity and anti-diabetic properties (Park, Storkson, Albright, Liu & Pariza, 1999) and the *cis*-9, *trans*-11 isomer predominantly anti-carcinogenic (McGowan et al., 2013; Mohammadzadeh, Faramarzi, Mahdahvi, Nasirimotlagh & Jafarabadi, 2013). However, much of the evidence surrounding the health benefits of CLA has been gathered from animal studies, with very little research conducted *in-vivo* with human subjects (Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006). CLA has also been shown to decrease proliferation and increase

apoptosis of certain cancer cells *in-vitro*, including colon cancer (Beppu et al., 2006), liver cancer (Igarashi & Miyazawa, 2000), and breast cancer (Shultz, Chew & Seaman, 1992) cells.

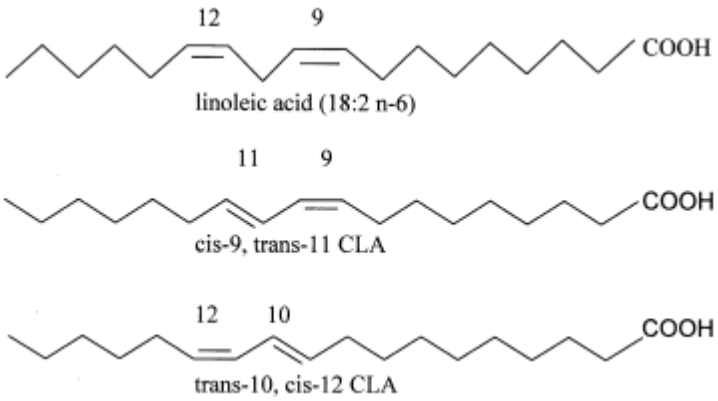

Figure 1. The structure of linoleic acid compared to the two main isomers of CLA, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA (Evans et al., 2002).

Synthesis of Conjugated Linoleic Acid in the Rumen

Kepler, Hirons, McNeill and Tove (1966) found that *cis*-9, *trans*-11 CLA was derived from the incomplete bio-hydrogenation of polyunsaturated LA to saturated stearic acid in the gut of ruminant animals. This chemical process was found to be carried out by bacteria, known as *Butyrivibrio fibrisolvens*, and is responsible for the vast majority of CLA production (Kepler et al., 1966). Chin, Storkson, Liu, Albright, and Pariza (1994) state that a small amount of *cis*-9, *trans*-11 CLA is absorbed directly into surrounding tissue, whereas most is hydrogenated at the 9-position in order to yield vaccenic acid (Harfoot & Hazlewood, 1988). The *cis*-9, *trans*-11 isomer can be

synthesised from vaccenic acid by the action of the Δ9-desaturase enzyme in the mammary and adipose cells of ruminants (Lawson, Moss & Givens, 2001).

Isomers of Conjugated Linoleic Acid

Although *cis*-9, *trans*-11 CLA is the predominant isomer found in natural sources, it is not the only important isomer, as *trans*-10, *cis*-12 is found in CLA that has been manufactured from LA (Kim et al., 2016). CLA isomers can be synthesised in the laboratory from LA, or oils high in LA, such as sunflower, safflower, and soybean (Sehat et al., 1998). Synthetic CLA isomers consist of 40-41% *cis*-9, *trans*-11, 44-45% *trans*-10, *cis*-12, and 5-10% *trans*-*trans* isomers (Silva, 2014). This preparation of CLA is often termed CLA mixture, or CLA 50:50 (Kim et al., 2016). Naturally occurring CLA is present at very low concentrations, and therefore CLA mixture is used for most research purposes (Kim et al., 2016). Both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA have different biological roles (Bhattacharya et al., 2006), and the less common *trans*-*trans* isomers have been found to exhibit anti-cancer (Beppu et al., 2006), anti-inflammation (Lee, Lim, Park & Kim, 2009) and hypocholesterolaemic (Gilbert, Gadang, Proctor, Jain & Devareddy, 2011) properties.

Dietary Sources of Conjugated Linoleic Acid

Research from Chin, Storkson and Pariza (1993) showed that the *cis*-9, *trans*-11 CLA isomer accounted for 73-94% total CLA in milk, dairy products, meat and processed meat. CLA content of meat can range from 5.6mg/g fat in lamb to 2.7mg/g fat in veal, with concentrations much lower in seafood (>0.6mg/g fat) and plant oils

(>0.7mg/g fat) (Chin et al., 1992). Mushtaq, Mangiapane and Hunter (2010) determined the CLA content of 112 UK food stuffs and found that processed cheese slices contained the highest concentration of *cis*-9, *trans*-11 CLA, at 7.3mg/g fat. It was suggested that the high CLA content of processed cheese slices was due to heat treatment during the food manufacturing process, and the addition of whey protein, which has been shown to increase CLA content in cheese (Shantha, Decker & Ustunol, 1992).

Dietary intake of CLA can vary, depending on individual diets, food processing, and cattle feeding (Chin et al., 1992). Mushtaq et al. (2010) estimate that dietary intake of CLA from natural sources in the UK is 97.5 mg/d, compared with the estimated 200 mg/d for men and 93-151 mg/d for women in the USA (Herbel, McGuire, McGuire & Shultz, 1998).

Anti-Obesity Effects of Conjugated Linoleic Acid

Arguably, the focus of CLA in scientific research has been its anti-obesity effects (Kim et al., 2016). Meta-analyses of human clinical studies have concluded that CLA supplementation has led to improvements in body mass index (BMI) and abdominal adiposity, when supplemented at 3.2-3.4g/d for at least 6 months (Whigham, Watras & Schoeller, 2007; Schoeller, Watras & Whigham, 2009). However, a meta-analysis from Onakpoya, Posadzki, Watson, Davies and Ernst (2012) concluded that evidence from randomised controlled trials was not enough to convincingly show that CLA had any effects on body composition in the long-term. It should also be noted that *in-vivo* human studies are rare, and the strongest evidence linking CLA to its anti-obesity

effects is from animal studies, specifically mice and rats (Bhattacharya et al., 2006). Park et al. (1997) tested the effects of CLA on body composition in mice and found that those fed a CLA-supplemented diet exhibited 60% less body fat than those fed a controlled diet. It is believed that the mechanisms behind CLA and anti-obesity in animals involves an increase in energy expenditure and fat utilisation, and a decrease in lipid storage, lipid synthesis, and adipogenesis in adipocytes (Park & Pariza, 2007; Park et al., 1997). It is specifically *trans*-10, *cis*-12 CLA that has been linked to improvements in body composition, whereas *cis*-9, *trans*-11 CLA has been shown to have no effects (Park et al., 1999).

Anti-Carcinogenic Effects of Conjugated Linoleic Acid

In addition to CLA's proposed anti-obesity health benefits, there has also been evidence linking CLA with the treatment and prevention of cancer (Kim et al., 2016). As with the anti-obesity research, most of the evidence gathered in this area is from animal-based studies. Ha et al. (1987) tested the effects of CLA on mice, and found that those treated with CLA had 50% less tumour incidence than those treated with pure LA. More recently, a small amount of human studies have investigated the effects of CLA intake on tissue CLA and cancer incidences, yet the findings were inconsistent (Bhattacharya et al., 2006). There are even fewer clinical trials involving the administration of CLA to cancer patients (Kim et al., 2016). A clinical trial conducted by McGowan et al. (2013) investigated the effects of CLA on the proliferation of breast cancer tumours. It was hypothesised that the administration of CLA would inhibit the expression of markers related to fatty acid synthesis in tumour tissue, and thus aid the

suppression of tumour proliferation. McGowan et al. (2013) provided 7.5g/d CLA mixture to 24 women with Stage I-III breast cancer, at least 10 days before their surgery. Using an immunohistochemical staining scoring system, it was shown that there was a significant decrease (p=.003) in the expression of Spot 14 (S14), a breast cancer tumour proliferation marker associated with lipid metabolism. In 11 of the 13 women with the most advanced tumours, and with the highest immunohistochemical staining scores, the supplementation of CLA was shown to decrease S14 expression, and thus lower their staining scores. In patients with less advanced tumours, and a lower staining score, CLA was found to have no effect on S14 expression. Therefore, it was suggested that CLA supplementation was more effective in the later stages of cancer when considering S14 expression. McGowan et al. (2013) propose that this score decrease suggests that the initial metabolic status of breast cancer cells may affect their response to CLA supplementation. Based on their observations, it was concluded that CLA may be used in conjunction with current breast cancer treatments.

A clinical trial from Mohammadzadeh et al. (2013) investigated the effects of CLA on rectal cancer patients undergoing chemoradiotherapy (CRT) in Tabriz, Iran. The experiment was a randomised, double-blind, placebo-controlled trial with 32 participants (18 male and 14 female). Participants were hospital patients with either stage II (n=12) or stage III (n=20) rectal cancer. Patients were given 3g/d of either CLA mixture (containing equal quantities of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) or placebo sunflower oil capsules, to take every day for six weeks. Results revealed that in comparison to the placebo group, patients consuming CLA had significantly improved biomarkers of inflammation (p=.004) and of angiogenesis and tumour invasion

(*p*=.004), indicated by levels of tumour necrosis factor α (TNF-α) and matrix metalloproteinase-2 (MP-2) enzyme, respectively. Angiogenesis is the growth of new blood vessels, allowing tumour growth through the deliverance of nutrients (Murukesh, Dive & Jayson, 2010). Angiogenesis biomarker discoveries can aid the treatment of rectal cancer, as they allow for the identification of cancer cells, and therefore the use of angiogenesis inhibitors (Mousa, Salem & Mikhail, 2015). Mohammadzadeh et al. (2013) concluded that CLA supplementation may provide complimentary treatment by reducing tumour invasion, and resistance to cancer treatment, in rectal cancer patients.

Conjugated Linoleic Acid *In-vitro*

CLA has been used for *in-vitro* studies using immortal cancer cell lines (Kim et al., 2016). Immortalised cell lines are developed from a single cell and have a uniform genetic makeup (Holliday & Speirs, 2011). They proliferate indefinitely, and are therefore cultured for many different types of *in-vitro* research. There is a body of research focusing on the *in-vitro* effects of CLA on a multitude of cancer cell lines, including colon cancer, breast cancer, and prostate cancer (Bhattacharya et al., 2006). Results all seem to be in agreement, and conclude that CLA either slows cancer cell proliferation (growth), or induces cell apoptosis (death) (Schønberg & Krokan, 1995). However, the evidence surrounding the effects of individual isomers on cancer cell proliferation is inconclusive (Maggiora et al., 2004). Shahzad et al. (2018) suggest that the effects of the different isomers, and their anti-proliferation mechanisms, are likely

to depend on the type and genetic makeup of the cancer cells themselves. Therefore, each cancer cell line may be affected differently with each isomer.

Palombo, Ganguly, Bistrian and Menard (2002) tested the effects of two commercial preparations of CLA, and three individual CLA isomers, on the proliferation of human colorectal (HT-29 and MIP-101) and prostate (PC-3) carcinoma cells. Each CLA preparation contained different major isomers, in varying proportions, with the first preparation (CLA-PN) consisting of 35% *cis*-9, *trans*-11 CLA and 36% *trans*-10, *cis*-12 CLA, and the second preparation (CLA-MAT) consisting of 75% *cis*-9, *trans*-11 CLA, 19% *cis*-9, *cis*-11 CLA and 1% *trans*-9, *trans*-11 CLA. Each of these two preparations of CLA were tested on HT-29, MIP-101, and PC-3 in concentrations of 178µM, 100µM, and 36µM. Palombo et al. (2002) also tested the effects of the *cis*-9, *trans*-11 CLA, *cis*-9, *cis*-11 CLA, and *trans*-10, *cis*-12 CLA individual isomers on the proliferation of HT-29, MIP-101, and PC-3, in concentrations of 100µM and 50µM.

HT-29 proliferation was completely inhibited (100%) by both CLA preparations at 178µM and 100µM, with a 50% inhibition at 36µM (p<.001). MIP-101 proliferation was inhibited by CLA-PN in a dose dependent manner at 178µM (p<.001), 100µM (p<.001) and 36µM (p<.005). However, CLA-MAT only significantly reduced MIP-101 cell proliferation at 178µM (p<.001), with no observed effects at either 100µM or 36µM. PC-3 proliferation was significantly inhibited by both CLA-PN (p<.001) and CLA-MAT (p<.001) at 178µM only, with no observed effects at 100µM or 36µM. CLA-PN was least effective at decreasing PC-3 cell proliferation (53%) when compared to HT-29 (100%) and MIP-101 (83%).

All individual isomers were significantly effective at reducing the proliferation of HT-29 cells at 100µM (p<.001) when compared with control groups, with 95% inhibition from *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, and 80% inhibition from *cis*-9, *cis*-11 CLA. Both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA significantly reduced proliferation by 50% at 50µM (p<.001). However, there were no observed effects on proliferation from *cis*-9, *cis*-11 CLA on the HT-29 cells. Both *cis*-9, *cis*-11 CLA and *cis*-9, *trans*-11 CLA were significantly effective (p<.001) at reducing MIP-101 cell proliferation, albeit to a lesser extent than *trans*-10, *cis*-12 CLA at both 100µM (95% reduction) and 50µM (80% reduction) (p<.001). PC-3 cell proliferation was significantly reduced by all isomers at 100µM (p<.001), yet not at 50µM, relative to the control group. The reduced proliferation of PC-3 cells by *cis*-9, *trans*-11 CLA was relatively low (32%) when compared with HT-29 (95%) and MIP-101 (49%) cells.

The results suggest that *trans*-10, *cis*-12 CLA exhibits the highest anti-proliferative effect on these cancer cell types, as it was found to be the most effective as an individual isomer, and as a main component of CLA-PN. Palombo et al. (2002) concluded that each of the cancer cells were affected in a dose- and isomer-dependent manner. The *trans*-*cis* and *cis*-*trans* CLA isomers were more effective at reducing, or completely inhibiting, cell proliferation than the *cis*-*cis* isomer. It was also concluded that net reduction in cancer cells is dependent upon the type and concentration of CLA, and the type of cancer cells targeted.

More recent studies support the findings of Palombo et al. (2002) and illustrate the varying effects of different CLA isomers. Shahzad et al. (2018) studied the effects of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA on the proliferation, migration, and

invasion of two different ovarian cancer cell lines, SKOV-3 and A2780. SKOV-3 and A2780 were plated in 96-well culture plates and treated with either *trans*-10, *cis*-12 CLA or *cis*-9, *trans*-11 CLA over four time points: 24 hours, 48 hours, 72 hours, and 96 hours. *Trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA were added to the ovarian cancer cells in seven different concentrations: 0µM, 1µM, 5µM, 10µM, 15µM, 20µM and 25µM. Results showed that all concentrations of *cis*-9, *trans*-11 CLA had no observed effect on the proliferation or viability of SKOV-3 and A2780 cells. On the contrary, *trans*-10, *cis*-12 CLA significantly ($p<.001$) reduced the proliferation of both SKOV-3 (90%) and A2780 (60%) cells. Results also showed that both *cis*-9, *trans*-11 CLA and t*rans*-10, *cis*-12 CLA had no effect on apoptosis in either cell line. Shahzad et al. (2018) concluded that *trans*-10, *cis*-12 CLA should be considered for the treatment of ovarian cancer.

Beppu et al. (2006) tested different isomers of CLA on three colon cancer cell lines, CaCo-2, HT-29, and DLD-1. The cultured cells were treated with *trans*-9, *trans*-11 CLA, *cis*-10, *trans*-12 CLA, *cis*-9, *trans*-11 CLA, and *cis*-9, *cis*-11 CLA, in concentrations ranging from 50µM to 200µM. The treated cells were incubated for 24 hours, 48 hours, and 72 hours, and tested for viability using an absorbance reader. The viability of the CaCo-2 cells was most strongly affected by the *trans*-9, *trans*-11 CLA isomer, which when compared to the control cells, reduced cell viability by 68% at 24 hours, 90% at 48 hours, and 98% at 72 hours. In comparison, the *cis*-9, *trans*-11 CLA isomer reduced cell viability by 20% at 24 hours, 35% at 48 hours, and 45% at 72 hours, when compared to the viability of control cells. *Trans*-9, *trans*-11 CLA was found to be most effective at reducing cell viability at a concentration of 200µM, however cell viability

was also reduced (<20%) with concentrations of 100µM and 50µM. The viability of the HT-29 and DLD cells was significantly reduced (p<.001) by the *trans*-9, *trans*-11 isomer only, with HT-29 at 58% of control cells and DLD at 68%. These findings conflict the evidence of Palombo et al. (2002), who found that HT-29 cell growth was inhibited up to 95% by *cis*-9, *trans*-11 CLA and up to 80% by *cis*-9, *cis*-11 CLA. Beppu et al. (2006) found that *trans*-9, *trans*-11 CLA significantly induced apoptosis (p<.001) in the CaCo-2 cells at concentrations of 50µM, 100µM and 200µM. Beppu et al. (2006) concluded that all CLA isomers acted in a dose- and time-dependent manner, with the *trans*-9, *trans*-11 CLA isomer showing the strongest anti-carcinogenic activity, yet state that the exact mechanism behind its actions is unknown. Proposed mechanisms involve increased lipid peroxidation, alteration of cellular fatty acid composition, and regulation of certain gene expressions (Beppu et al., 2006).

Despite there being evidence that *trans*-10, *cis*-12 CLA and *trans*-9, *trans*-11 CLA isomers have the most potent anti-proliferative effects (Shahzad et al., 2018; Palombo et al., 2002; Beppu et al., 2006), there is also evidence supporting the anti-carcinogenic properties of *cis*-9, *trans*-11 CLA. Liu et al. (2002) treated mammary cancer (MCF-7) *in-vitro* with 25µM, 50µM, 100µM and 200µM *cis*-9, *trans*-11 CLA for 24 hours and 48 hours. After 48 hours, cell growth was inhibited by 27% at 25µM, 35% at 50µM, 91% at 100µM, and 93% at 200µM. It was suggested by Liu et al. (2002) that the actions of *cis*-9, *trans*-11 CLA involved the cell cycle arrest of MCF-7. The mechanism behind the action was further investigated by Liu et al. (2002), who tested *cis*-9, *trans*-11 CLA on gastric adenocarcioma cells (SGC-7901), in concentrations of 25µM, 50µM, 100µM, and 200µM, for 24 hours and 48 hours. After 48 hours, cell

growth rates were inhibited by 6% at 25µM, 20% at 50µM, 76% at 100µM, and 82% at 200µM. There was also a significantly greater incidence of apoptosis at all concentrations when compared to control cells (p<.01). From their results investigating biomarkers of proliferation (Ki67) and apoptosis (Bcl-2), and apoptosis antigens (Fas) and genes (c-myc), Liu et al. (2002) concluded that *cis*-9, *trans*-11 CLA blocked the cell-cycle at the G_0/G_1 phase. CLA treated cells expressed significantly less Ki67 than control cells (p<.01), in a dose-dependent manner. However, Liu et al. (2002) state that the exact mechanisms behind the action of *cis*-9, *trans*-11 CLA are not fully understood, and require more research.

Proposed mechanisms behind CLA effects

Cho et al. (2003) found that CLA mixture inhibited both cell proliferation, DNA synthesis, and ErbB3 signalling in HT-29 human colon cancer cells. ErbB3 is part of a family of epidermal growth factor receptors (EGFR), which are involved in several processes, including cell proliferation and cell differentiation (Nicholson, Gee & Harper, 2001). Over expression of one receptor, ErbB2, has been linked with ErbB3 and the development of colon cancer, with both proteins expressed in the HT-29 cell line at high levels (Cho et al., 2003). The mechanisms behind the CLA regulation of ErbB2 and ErbB3 were not investigated by Cho et al. (2003), yet it has been suggested that CLA alters gene transcription by acting as a ligand for PPARγ. Ligands for PPARγ have been found to induce cell apoptosis and have anti-proliferative effects on many carcinoma cell lines (Elstner et al., 1998; Kwon, Jovanovic, Serfas, Kiyokawa & Tyner, 2002).

The A431 epidermoid carcinoma cell line

Epidermoid carcinoma, also known as squamous cell carcinoma, is one of the largest sub-sets of cancer (Berman, 2004). Epidermoid carcinoma is a type of cancer that affects the cells forming the surface of the skin, the lining of hollow organs, and the lining of the digestive and respiratory tracts (Melo Neto et al., 2013). Epidermoid carcinoma comprises several cancers, such as skin, lung, and vaginal, each associated with different symptoms, prognosis and response to treatment (Yu, Yang, Hu & Yan, 2009). The A431 epidermoid carcinoma cell line originates from the vulva of an 85-year old female cancer patient (Barnes, 1982). A431 cells are commonly used in cancer studies due their high expression of epidermal growth factor receptors (EGFR) (Bhatia, Agarwal & Agarwal, 2001).

Conclusion

The literature surrounding the health benefits of CLA is extensive. Although much of the original research was conducted using animal subjects, more recent studies have used human cells for *in-vitro*, and human patients for hospital-based clinical trials. Most of the evidence illustrates that CLA exhibits beneficial effects on human health. Findings from anti-obesity research have illustrated that supplementation with CLA leads to a short-term significant reduction in BMI, total body weight and abdominal adiposity. Evidence from clinical trials suggest that supplementation with CLA can lead to a reduction in tumour proliferation in women in the later stages of breast cancer. Further research has shown that when compared to placebo, CLA supplementation improves biomarkers of inflammation and tumour

invasion in rectal cancer patients in Iran. Extensive *in-vitro* research has focused on the anti-proliferative effects of commercial CLA preparations and individual CLA isomers on cancer cells. There is a large body of evidence illustrating the anti-proliferative effects of CLA on colon, prostate, breast and ovarian cancer. Certain studies highlight the effects of individual isomers, specifically *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA, for their anti-proliferative properties. All studies thus far agree that the *trans*-10, *cis*-12 CLA isomer has the strongest anti-proliferative properties. However, regarding the *cis*-9, *trans*-11 CLA isomer, evidence is conflicting. Certain studies have found significant effects of *cis*-9, *trans*-11 CLA on the reduced proliferation of breast and gastric cancer cells. Moderate effects have been found from studies involving colon and prostate cancer, and no effects were found in ovarian cancer. However, most *in-vitro* studies have concluded that the effects of individual CLA isomers are specific to the cancer cells that are being treated. All *in-vitro* studies show that CLA works in a dose- and time-dependent manner, yet the exact mechanisms behind the actions remain inconclusive, with theories proposing lipid peroxidation, alteration of cellular fatty acid composition, and regulation of certain gene expressions.

To date, *in-vitro* CLA research has focused on multiple cancer cell lines, including HT-29, MIP-101, PC-3, SKOV-3, A2780, CaCo-2, DLD-101, and MCF-7. To our knowledge, there are currently no studies that have tested the effects of CLA on the proliferation of A431 epidermoid carcinoma cells, specifically the *cis*-9, *trans*-11 CLA isomer. Some of the evidence surrounding the effects of this isomer is conflicting, however its anti-proliferative effects have been shown when tested on colon, prostate, and gastric cancer, with the strongest effects shown on breast cancer.

The current study will test the effects of four different concentrations of *cis*-9, *trans*-11 CLA on the proliferation of A431 epidermoid carcinoma cells over 48 hours. Based on previous literature, it is hypothesised that *cis*-9, *trans*-11 CLA will slow the proliferation of A431 cells in a time- and dose-dependent manner.

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The Effects of *cis*-9, *trans*-11 Conjugated Linoleic Acid on the Proliferation of A431 Epidermoid Carcinoma Cells

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The journal deemed suitable for this article is *The Journal of Nutritional Biochemistry*. The Journal of Nutritional Biochemistry is devoted to advancements in nutritional sciences, presenting experimental nutrition research in relation to biochemistry, molecular biology, toxicology, and physiology. This article links the area of nutrition with biochemistry and molecular biology, through the examination of the effects of conjugated linoleic acid on the proliferation of cultured A431 epidermoid carcinoma cells.

Abstract

Background and aims

The aim of this study was to investigate the effects of varying concentrations of the *cis*-9, *trans*-11 isomer of conjugated linoleic acid (CLA) on the proliferation of A431 epidermoid carcinoma cells across three separate time points. A strong collection of *in-vitro* studies illustrate the anti-carcinogenic properties of CLA isomers on colon cancer, prostate cancer, breast cancer, and ovarian cancer. Currently, there is no research investigating the effects of CLA on the proliferation of epidermoid carcinoma, specifically the *cis*-9, *trans*-11 CLA isomer.

Methods

Four concentrations of *cis*-9, *trans*-11 CLA (25µM, 50µM, 100µM and 200µM) were tested on cultured A431 epidermoid carcinoma cells. Proliferation was measured using an MTS assay after zero hours, 24 hours, and 48 hours.

Results

All treated cells were found to significantly proliferate between zero and 24 hours. There were no significant differences in proliferation found between the treated cells and the control cells.

Conclusion

The findings of this study do not support previous *in-vitro* studies on the anti-proliferative effects of *cis*-9, *trans*-11 CLA. In future, cells should be incubated with the

treatment for a longer period, in order to observe potentially anti-proliferative effects. However, as a result of the current findings, *cis*-9, *trans*-11 CLA should not be used as a treatment for, or in conjunction with treatment for, epidermoid carcinoma.

1. Introduction

Conjugated linoleic acid (CLA), an isomer of linoleic acid (LA), is a naturally occurring 18-carbon polyunsaturated fatty acid found in the meat and dairy products of ruminant animals (Chin, Liu, Storkson, Ha & Pariza, 1992). There are 28 positional and geometrical isomers of CLA, with double bonds arranged in *cis*-*trans*, *trans*-*cis*, *cis*-*cis*, or *trans*-*trans* configurations (Kepler, Hirons, McNeill & Tove, 1966). CLA is produced in the gastrointestinal tract of ruminant animals, by bacteria known as *Butyrivibrio fibrosolvens*, or is synthesised in ruminant mammary and adipose cells by the action of the Δ9-desaturase enzyme (Lawson, Moss & Givens, 2001).

The major isomer found in food is *cis*-9, *trans*-11 CLA, accounting for 73-94% of CLA found in dairy products and meat (Chin et al., 1992). Mushtaq, Mangiapane and Hunter (2010) estimated dietary intake of CLA to be 97.5mg/d in the UK.

Early research into the effects of CLA revealed its anti-carcinogenic properties (Ha, Grimm & Pariza, 1987), and positive effects on body composition (Park et al., 1997). However, many of the original studies were conducted on mice and rats (Kim, Kim, Kim & Park, 2016). More recently, human *in-vitro* and *in-vivo* studies have supported the original findings and identified key isomers and the mechanisms behind their actions (Park et al., 1997). CLA has been shown to lead to an increase in cell apoptosis (death) and a slow in proliferation (growth), however the exact mechanisms are not fully understood (Kim et al., 2016). It has been proposed that the anti-carcinogenic effects of CLA involve lipid peroxidation, alteration of fatty acid composition, and the regulation of gene expression (Beppu et al., 2006).

In-vivo studies have illustrated that CLA supplementation reduced tumour proliferation in females with late stage breast cancer (McGowan et al., 2013), and increased biomarkers of inflammation in rectal cancer patients (Mohammadezah, Faramarzi, Mahdahvi, Nasirimotlagh & Jafarabadi, 2013). Conclusions from these studies suggest that CLA should be used in conjunction with current cancer treatments. However, there are very few *in-vivo* studies investigating the anti-carcinogenic effects of CLA, and most of the evidence is derived from *in-vitro* research.

In-vitro studies have highlighted the anti-carcinogenic effects of the two main CLA isomers: *cis*-9, *trans*-11 and *trans*-10, *cis*-12. Strong anti-proliferative effects of *cis*-9, *trans*-11 CLA have been found from studies focusing on breast cancer (Liu et al., 2002) and gastric cancer (Liu et al., 2002), and moderate effects have been found in colon (Beppu et al., 2006; Palombo, Ganguly, Bristrian & Menard, 2002) and prostate cancer (Palombo et al., 2002). Shahzad et al. (2018) concluded from their investigation using ovarian cancer cells than *cis*-9, *trans*-11 CLA had no effect on cell proliferation for these specific cell lines. However, Palombo et al. (2002) state that the effects of the CLA isomers are dependent upon the type of cancer cells they are tested on. Findings from *in-vitro* studies show that *cis*-9, *trans*-11 CLA works in a time- and dose-dependent manner in terms of anti-proliferative effects (Liu et al., 2002; Palombo et al., 2002).

To date, *in-vitro* CLA research has focused on multiple cancer cell lines, including HT-29, MIP-101, PC-3, and MCF-7. To our knowledge, there are currently no studies that have investigated the effects of CLA on the A431 epidermoid carcinoma cell line. Epidermoid carcinoma is cancer of the cells that form the surface of the skin,

the lining of hollow organs, and the lining of the digestive and respiratory tracts (Melo Neto et al., 2013). Epidermoid carcinoma is one of the largest sub-sets of cancer, and includes skin cancer, lung cancer, and vaginal cancer (Berman, 2004). The A431 epidermoid carcinoma cell line originates from the vulva of an 85-year old female cancer patient (Barnes, 1982).

The current study will test the effects of four different concentrations of *cis*-9, *trans*-11 CLA on the proliferation of A431 epidermoid carcinoma cells over 48 hours. Based on previous literature, it is hypothesised that *cis*-9, *trans*-11 CLA will slow the proliferation of A431 cells in a time- and dose-dependent manner.

2. Methods and Materials

2.1 Materials

Epidermoid carcinoma cell line, A431, was purchased from the European Collection of Authenticated Cell Cultures (ECACC). The *cis*-9, *trans*-11 CLA isomer, and MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric proliferation assay were purchased from Sigma Aldrich.

2.2 Cell culture conditions

A431 cells were cultured in Dulbecco's modified eagle medium (DMEM) (500ml) supplemented with 50ml (10%) foetal bovine serum (FBS) and 5ml (1%) penicillin, streptomycin and glutamine mixture. Cell cultures were maintained within an incubator at 37°C and 5% CO_2, and passaged when 80% confluent.

2.3 In-vitro CLA treatment

Human epidermoid carcinoma cells were cultured and seeded at a density of 1 x 10^4 cells/well in 96-well microtiter plates. The cells were cultured in 100µL media/well and incubated for 24 hours at 37°C and 5% CO_2. Stock solutions of CLA were prepared in industrial methylated spirits (IMS) at a concentration of 200,000µM. A second stock was prepared in media from the IMS stock at a concentration of 2,000µM, and diluted to the final concentrations of 25µM, 50µM, 100µM and 200µM. Each concentration was plated in triplicate. In addition, carrier control cells were also plated, containing cells and solvent, without *cis*-9, *trans*-11 CLA. Vehicle control cells were also plated, containing only media and MTS reagent in order to test the effects of the MTS reagent on the absorbance reading. Plates were incubated for the desired time period (0 hours, 24 hours or 48 hours), and 20µL/well MTS reagent was added directly onto the cells, and incubated for a further 2 hours. See Appendix A for full protocol.

2.4 Cell proliferation assessment

Cell proliferation was assessed using colorimetric MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. MTS tetrazolium compound reduction generated a coloured formazan product in viable cells, which was quantified by measuring absorbance at OD=490nm. Higher absorbance readings indicated viable cells, and therefore proliferation.

2.5 Statistical Analysis

Descriptive and inferential statistics were generated using Statistical Package for Social Scientists software (SPSS, Inc., version 24.0, Chicago, IL). A two-way mixed-model analysis of variance (ANOVA) with Tukey post-hoc tests was conducted to examine the differences between the means of the between-subject variables (concentration of *cis*-9, *trans*-11 CLA) and the means of the within-subject variables (incubation time period). Homogeneity of variance was examined using Levene's test. The significance level for all tests was set at $p<.05$.

3. Results

No significant interaction was found between the concentration of *cis*-9, *trans*-11 CLA and incubation time on the proliferation of A431 epidermoid carcinoma cells ($p=.06$). Cell proliferation was significantly increased by time ($p<.001$). This difference was found to be between the zero hour and 24-hour plate readings ($p<.001$). Cell proliferation was found to significantly increase at 25µM from zero hours (M = .82 ± .03nm) to 24 hours (M = 1.13 ± .03nm, p = .001), at 50µM from zero hours (M = .88 ± .05nm) to 24 hours (M = 1.16 ± .06nm, p = .04), at 100µM from zero hours (M = .84 ± .06nm) to 24 hours (M = 1.19 ± .06nm, p = .004), at 200µM from zero hours (M = .90 ± .07nm) to 24 hours (M = 1.19 ± .05nm, p = .006), and control cells at zero hours (M = .82 ± .08nm) to 24 hours (M = 1.11 ± .09nm, p = .015). There were no significant differences at any concentration between the 24-hour and 48-hour plate readings ($p=.21$).

Cell proliferation was not significantly affected by concentration. Treating epidermoid carcinoma cells with *cis*-9, *trans*-11 CLA at a concentration of 25µM did not significantly affect proliferation when compared to controls (see Figure 1).

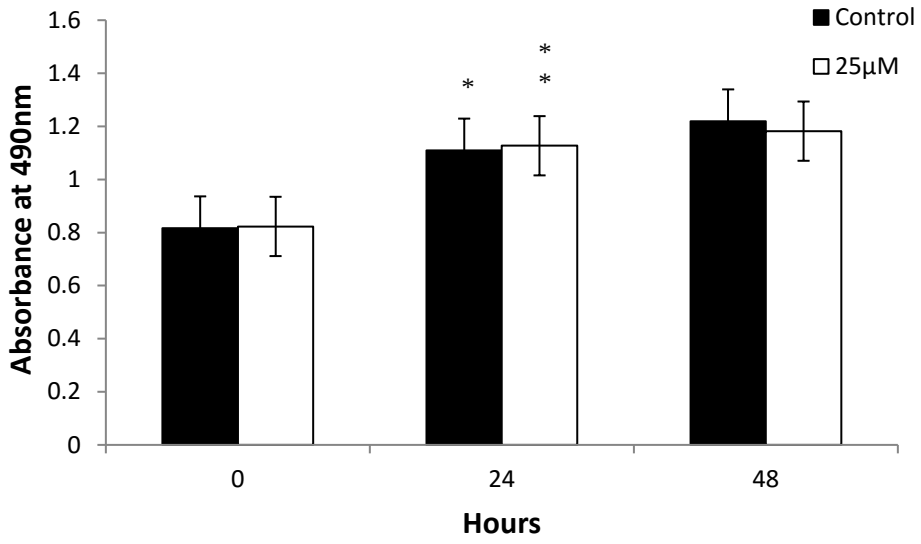

Figure 1. The mean absorbance of control cells and *cis*-9, *trans*-11 CLA treated cells, at a concentration of 25µM, across three time points. Error bars represent ± SE. The asterisk indicates a significant increase in proliferation between 0 hours and 24 hours (* = $p \leq .05$, **= $p \leq .01$).

There were no significant differences found between the absorbance of cells treated with 25µM *cis*-9, *trans*-11 CLA and control cells at zero hours ($p = 1.00$) or 24 hours ($p = .99$). At 48 hours, the absorbance of cells treated with 25µM *cis*-9, *trans*-11 CLA ($M = 1.19 \pm .06$nm) was not significantly different to the absorbance of control cells ($M = 1.22 \pm .06$nm, $p = .88$).

Treating A431 epidermoid carcinoma cells with *cis*-9, *trans*-11 CLA at a concentration of 50µM did not significantly increase or decrease proliferation when compared to controls (see Figure 2).

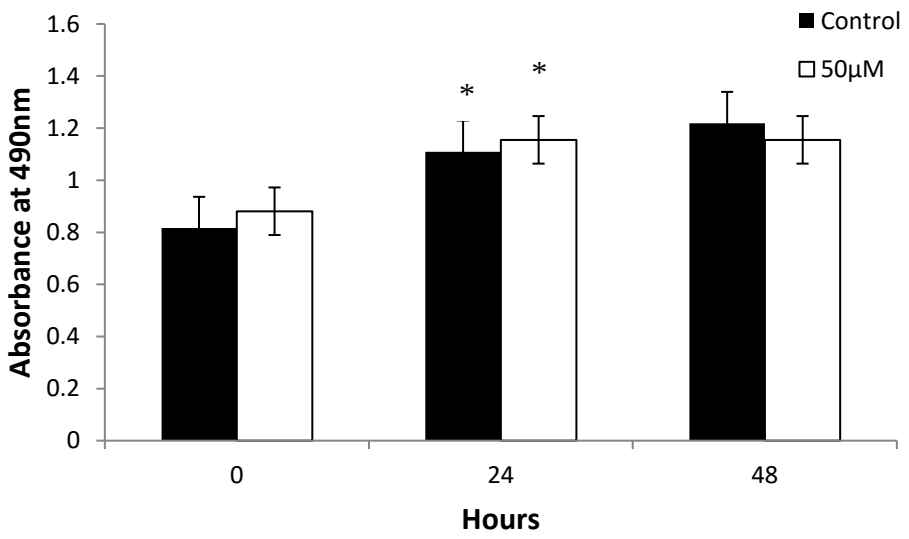

Figure 2. The mean absorbance of control cells and *cis*-9, *trans*-11 CLA treated cells, at a concentration of 50µM, across three time points. Error bars represent ± SE. The asterisk indicates a significant increase in proliferation between 0 hours and 24 hours (* = $p \leq .05$).

There were no significant differences between the absorbance of cells treated with 50µM *cis*-9, *trans*-11 CLA and control cells at zero hours ($p = .71$) or 24 hours ($p = .88$). At 48 hours, the absorbance of cells treated with 50µM *cis*-9, *trans*-11 CLA ($M = 1.15 \pm .03$nm) was not significantly different to the control cells ($M = 1.22 \pm .06$nm, $p = .53$).

Treating A431 epidermoid carcinoma cells with *cis*-9, *trans*-11 CLA at a concentration of 100µM did not significantly increase or decrease proliferation when compared to controls (see Figure 3).

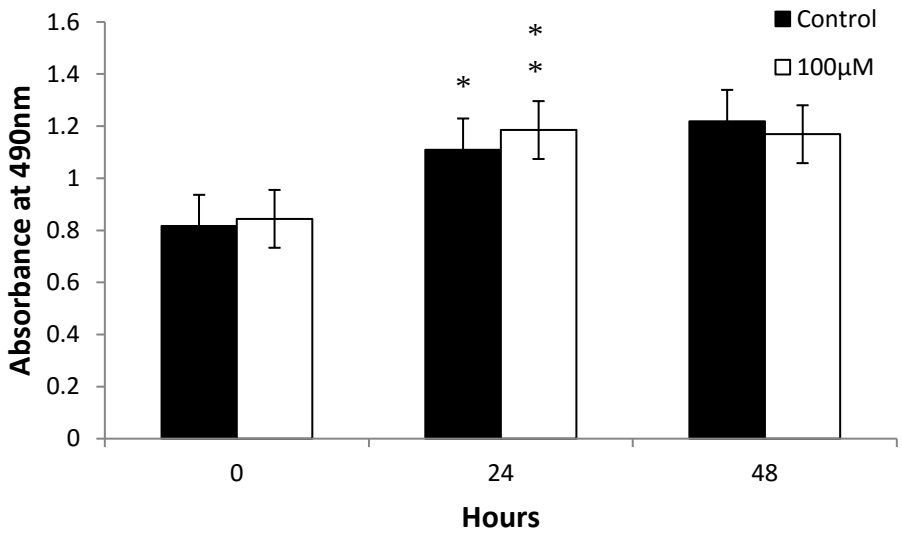

Figure 3. The mean absorbance of control cells and *cis*-9, *trans*-11 CLA treated cells, at a concentration of 100µM, across three time points. Error bars represent ± SE. The asterisk indicates a significant increase in proliferation between 0 hours and 24 hours (* = $p \leq .05$, **= $p \leq .01$).

There were no significant differences between the absorbance of cells treated with 100µM *cis*-9, *trans*-11 CLA and control cells at zero hours ($p = .98$) or 24 hours ($p = .57$). At 48 hours, the absorbance of cells treated with 100µM *cis*-9, *trans*-11 CLA ($M = 1.17 \pm .04$nm) was not significantly different to the control cells ($M = 1.22 \pm .06$nm, $p = .73$).

Treating A431 epidermoid carcinoma cells with *cis*-9, *trans*-11 CLA at a concentration of 200µM did not significantly increase or decrease proliferation when compared to controls (see Figure 4).

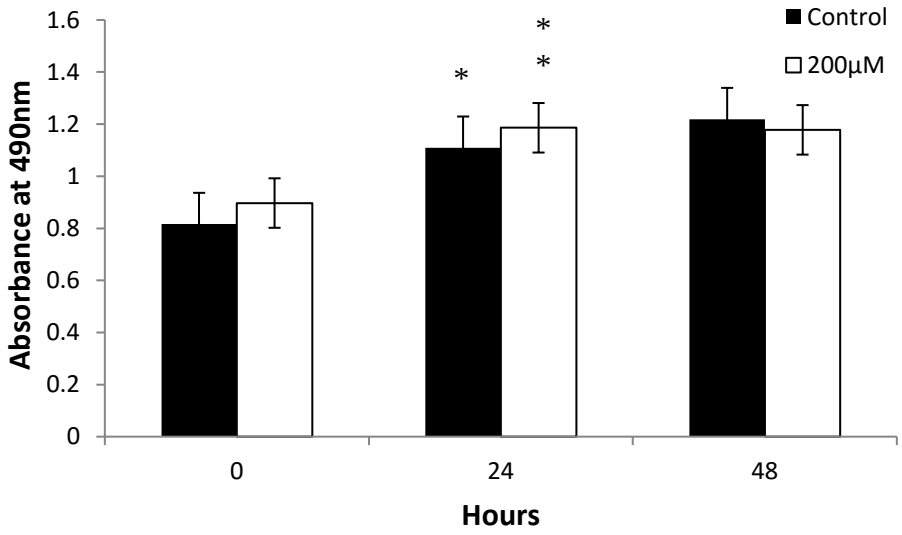

Figure 4. The mean absorbance of control cells and *cis*-9, *trans*-11 CLA treated cells, at a concentration of 200µM, across three time points. Error bars represent ± SE. The asterisk indicates a significant increase in proliferation between 0 hours and 24 hours (* = $p \leq .05$, **= $p \leq .01$).

There were no significant differences between the absorbance of cells treated with 200µM *cis*-9, *trans*-11 CLA and control cells at zero hours ($p = .53$) or 24 hours ($p = .55$). At 48 hours, the absorbance of cells treated with 200µM *cis*-9, *trans*-11 CLA ($M = 1.19 \pm .06$nm) was not significantly different to control cells ($M = 1.22 \pm .06$nm, $p = .84$).

When comparing each concentration across each time point, proliferation of the treated cells was found to slow in comparison to control cells (see Figure 5). However, no significant differences were found.

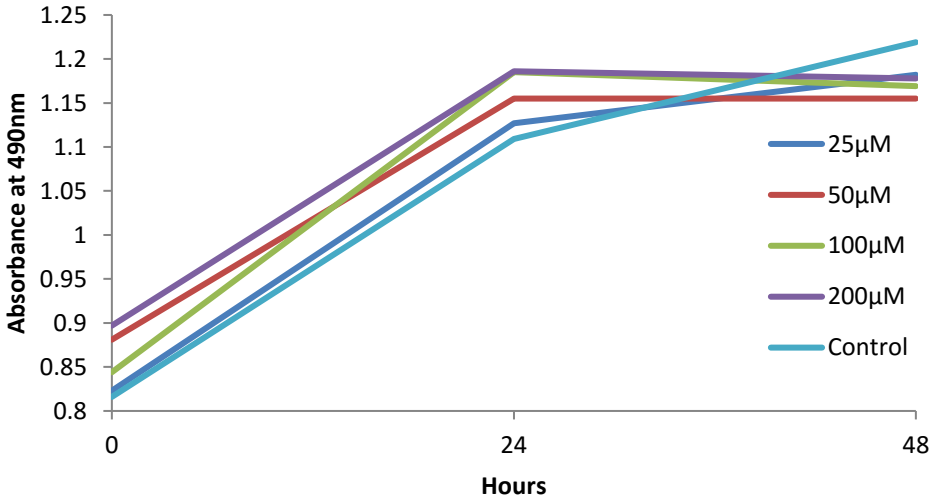

Figure 5. The overall trend in proliferation of the five concentrations of *cis*-9, *trans*-11 CLA across three time points.

4. Discussion

The results from this experiment reject the hypothesis that *cis*-9, *trans*-11 CLA would inhibit the proliferation of A431 epidermoid carcinoma cells in a time- and dose-dependent manner. There were no significant differences in proliferation found between control cells and any concentrations of *cis*-9, *trans*-11 CLA at any time point.

Contrary to the hypothesis, results showed that time, independent of concentration, lead to a significant increase in cell proliferation. The absorbance measured at 24 hours was significantly higher than absorbance measured at zero hours across all concentrations and control cells. The absorbance measured at 48

hours was not significantly different to the absorbance measured at 24 hours. Results show that concentration had no significant effect on the proliferation of epidermoid carcinoma cells. Growth was expected to slow with the addition of *cis*-9, *trans*-11 CLA, especially with the higher concentrations of 100µM and 200µM. Control cells were predicted to grow as normal and show the highest rate of proliferation. However, the evidence supported neither of these predictions.

There are currently no studies investigating the effects of *cis*-9, *trans*-11 CLA on the proliferation of epidermoid carcinoma cells, and the literature surrounding the *in-vitro* effects of *cis*-9, *trans*-11 CLA is conflicting, with evidence both supporting and refuting its anti-carcinogenic properties (Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006). When tested alone, *cis*-9, *trans*-11 CLA has shown significant anti-proliferative effects on cancer cells, as well as inducing apoptosis (Liu et al., 2002; Chujo et al., 2003). However, when tested in conjunction with other CLA isomers, *cis*-9, - *trans*-11 CLA has shown the least anti-carcinogenic effects (Palombo et al., 2002; Shahzad et al., 2018).

The findings from this study are in line with findings from Shahzad et al. (2018), who tested the effects of different CLA isomers on the proliferation of ovarian cancer cells (SKOV-3 and A2780). Cells were treated with varying concentrations (0µM to 25µM) of either *trans*-10, *cis*-12 or *cis*-9, *trans*-11 CLA, and incubated for 24 to 96 hours. From their results, Shahzad et al. (2018) concluded that *cis*-9, *trans*-11 CLA had no effect on either proliferation or apoptosis of either type of ovarian cancer cells. However, the *trans*-10, *cis*-12 isomer was found to significantly reduce proliferation and cell viability in both cell types.

The findings from the present study contradict evidence from Liu et al. (2002), focusing on breast cancer, and Liu et al. (2002), focusing on gastric cancer. Results from Liu et al. (2002), who tested the effects of *cis*-9, *trans*-11 CLA on the proliferation of MCF-7, a breast cancer cell line, showed that *cis*-9, *trans*-11 CLA significantly reduced the proliferation of MCF-7 cells in a time- and dose-dependent manner. The *cis*-9, *trans*-11 CLA dosage (25µM, 50µM, 100µM and 200µM), and the incubation time (24 and 48 hours), were exactly the same in the study from Liu et al. (2002) as they are in the present study. Further research from Liu et al. (2002) focused on the effects of *cis*-9, *trans*-11 CLA on the *in-vitro* proliferation of gastric adenocarcinoma cells (SGC-7901). Using the exact same dosage and incubation time as the present study, and of Liu et al. (2002), it was found that *cis*-9, *trans*-11 CLA significantly inhibited the growth of SGC-7901 cells in a time- and dose-dependent manner. This inhibition was found to be due not only to a slow in proliferation, but also an increase in apoptosis.

The findings from Liu et al. (2002) and Liu et al. (2002) are in line with those of Beppu et al. (2006) who tested different isomers of CLA on the proliferation of three colon cancer cells lines (CaCo-2, HT-29, and DLD-1). The *cis*-9, *trans*-11 CLA isomer was found to significantly decrease the proliferation of all three colon cancers in concentrations ranging from 50µM to 200µM, across incubation periods of 24, 48 and 72 hours. Although all isomers showed significant anti-proliferative actions, the effects of *cis*-9, *trans*-11 CLA were overshadowed by the effects of *trans*-9, *trans*-11 CLA and *cis*-10, *trans*-12 CLA.

The fact that *cis*-9, *trans*-11 CLA showed significant effects in the studies from Liu et al. (2002), Liu et al. (2002) and Beppu et al. (2006), yet showed no effects in the

study from Shahzad et al. (2018), nor the present study, could suggest that perhaps *cis*-9, *trans*-11 CLA is only effective in certain types of cancer. According to Palombo et al. (2002), the *in-vitro* proliferation of cancer cells is dependent upon the type and concentration of CLA, as the effectiveness of the different isomers are not equivalent, and function through different mechanisms. This idea is supported by Maggiora et al. (2004), who suggest that the difference in effects is dependent upon the chemical nature of the fatty acid, or on the malignancy of the tumour cells. This could explain the conflicting body of evidence surrounding *cis*-9, *trans*-11 CLA, and why significant anti-proliferative effects have been found in certain *in-vitro* studies (Liu et al., 2002; Beppu et al., 2006), yet not the present study.

Support for the anti-proliferative effects of other isomers was provided by Palombo et al. (2002), who tested the effects of *cis*-9, *trans*-11 CLA, *cis*-9, *cis*-11 CLA, and *trans*-10, *cis*-12 CLA on the growth of prostate cancer (PC-3) and two types of colon cancer (CaCo-2 and MIP-101) at varying concentrations and incubation times. Results showed that both the *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers were effective at slowing the proliferation of both CaCo-2 and MIP-101. Although all isomers showed anti-proliferative effects on the PC-3 cells, the actions of the *cis*-9, *trans*-11 CLA isomer were relatively low when compared to *cis*-9, *cis*-11 CLA and *trans*-10, *cis*-12 CLA.

In order to understand the potential anti-proliferative effects of *cis*-9, *trans*-11 CLA, it is important to understand the mechanisms behind its actions. Liu et al (2002) suggest that the actions of *cis*-9, *trans*-11 CLA involve cell cycle arrest, and speculated that *cis*-9, *trans*-11 CLA blocks the cell-cycle at the G_0/G_1 phase. Ochoa et al. (2004)

linked the actions of *cis*-9, *trans*-11 CLA with two enzymes, 5-LOX and COX-2. These enzymes are involved in arachidonic acid metabolism, an important factor in oncogenesis and the progression of cancer (Ochoa et al., 2004). In their study, Ochoa et al. (2004) demonstrated that *cis*-9, *trans*-11 CLA lead to significant decreases in 5-LOX and COX-2 expressions. Cho et al. (2003) suggest that CLA alters gene transcription by acting as a ligand for PPARγ. PPARγ ligands have been found to induce cell apoptosis and slow cell proliferation *in-vitro* (Elstner et al., 1998). Ochoa et al. (2004) suggest that the increased apoptosis associated with *cis*-9, *trans*-11 CLA is due to the increased expression of Bcl-2, an important anti-apoptotic oncogene product. However, it is clear from the literature that the exact mechanisms behind the action of *cis*-9, *trans*-11 CLA are not fully understood (Liu et al., 2002). The results from the present study indicate that these mechanisms were not used by *cis*-9, *trans*-11 CLA on the A431 cell line.

The main limitation of this study is that the treated cells were only incubated for 48 hours. An additional 24 hours incubation time could have led to significant differences in proliferation, as found by Chujo et al. (2003). Chujo et al. (2003) found that *cis*-9, *trans*-11 CLA significantly reduced the proliferation of MCF-7 breast cancer cells, only after 72 hours incubation. Furthermore, Maggiora et al. (2004) found that the inhibition of liver cancer cell proliferation was affected only after 48 hours of exposure, regardless of concentration used.

Another potential limitation of this study is that only one plate of cells was tested at each time point, providing only three readings for each concentration. These three readings may not have been accurate, and therefore could have affected results.

Although the present study is associated with some limitations, it should be remembered that this research was novel, and results add to the growing body of evidence showing that perhaps the *cis*-9, *trans*-11 CLA isomer may not be as anti-carcinogenic as previously thought (Shahzad et al., 2018).

Research into the use of CLA as an alternative treatment for cancer is growing, however findings are inconclusive as to the effects of different isomers (Ochoa et al., 2004). Results from *in-vivo* studies have led to the conclusion that CLA should be used in conjunction with current treatments for breast and rectal cancer (McGowan et al., 2013; Mohammadzadeh et al., 2013). However, if CLA is to be used in the treatment of cancer, its mechanisms must first be fully understood (Kim et al., 2016). In terms of the *cis*-9, *trans*-11 CLA isomer, there is currently no evidence to suggest any anti-proliferative effect on epidermoid carcinoma cells. Therefore, results from this study suggest that supplementation with *cis*-9, *trans*-11 CLA would not be an appropriate treatment for people suffering from epidermoid carcinoma.

Future studies in this area may benefit from focusing on the effects of the *trans*-10, *cis*-12 CLA isomer. This isomer has shown strong anti-proliferative effects in the treatment of colon, prostate and ovarian cancers (Palombo et al., 2002; Shahzad et al., 2018). Based on this past research, *trans*-10, *cis*-12 CLA may prove effective at slowing the proliferation of A431 cells. Any future research using the *cis*-9, *trans*-11 CLA on epidermoid carcinoma cells should consider a longer incubation time, as effects may only been seen after 72 hours (Chujo et al., 2003). Testing repeat plates may also give more valid results (Ibrahim et al., 2012).

In conclusion, *cis*-9, *trans*-11 CLA may be regarded as an anti-carcinogenic component of the diet, effectively reducing the proliferation of colon, breast and gastric cancer *in-vitro* (Palombo et al., 2002; Liu et al., 2002; Liu et al., 2002). However, in the case of epidermoid carcinoma cells, this particular isomer has shown no significant anti-proliferative effects. Despite the facts that the results from this study reject the main hypothesis, the findings add to the growing body of research that disputes the anti-carcinogenic and anti-proliferative effects of *cis*-9, *trans*-11 CLA. In future, the use of *tran*-10, *cis*-12 CLA may potentially exhibit anti-proliferative effects on epidermoid carcinoma cells, based on previous extensive *in-vitro* evidence. However, current findings illustrate that the growth of A431 cells is not significantly affected by CLA, and therefore CLA should not be considered as a treatment for epidermoid carcinoma.

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Appendix A

Cell proliferation protocol

MTS Assay Protocol

- Calculate the amount of media needed
- Remove media from fridge, aliquot calculated amount (8.4ml) and place in the water bath to warm up
- Warm trypsin and PBS in water bath
- Spray in media, waste bottle, pipettes, pipette tips, pipette tip waste bottle, a glass vial, and 4 x 96 well plates into the safety cabinet (spray in the whole packet of well plates and open within the hood).
- Passage and count cells using haemocytometer (refer to passage protocol).
- *Calculations*:
    - 10^4 cells per well needed, and 21 wells per plate (4 plates, so 84 wells to tal)
    - $(21 \times 10^4) \times 4 = 84 \times 10^4$ cells
    - $(21 \times 10^4) \times 4 = 8.4$mL media
- Divide 84×10^4 by the amount of cells counted in the passage e.g. what we want/what we have.

The answer is the amount of cells (in µL) that should be added to 8.4mL of media, to give $1 \times 10^4/100\mu L$.

- Pipette the calculated amount of cells into the 8.4mL of media and mix
- Add 100µL of the cell/media solution to each well of each plate (as per template) (4 plates; 21 wells each) using a single-tipped pipette.
- Make sure to invert the tube every 2ml or so, to stop the cells settling (put the cap on first!)
- Once all wells have been seeded, incubate the plates for 24 hours in the incubator at 37^{o}C and 5% CO_2.

Make the ethanol stock in preparation for the next day:

- Remove the CLA from the freezer and allow to defrost
- Spray CLA into the hood
- Pipette 1mL of IMS (ethanol) into a glass vial
- Pipette 6.2µL of CLA into the ethanol
- Vortex for 2 minutes
- Store overnight in a freezer at -20^{o}C

After 24 hours

- Spray in waste bottle, pen, pipettes, pipette tips, pipette tip waste bottle, 4 x 5ml bijous and ethanol stock.
- Prepare the media stock solutions for each concentration:
    - Remove media from the fridge and spray into the cabinet
    - Aliquot 10mL of media and then warm in the water bath
    - Once warmed, spray back into the cabinet
    - Label 4 5ml bijou tubes “200mM”, “100mM”, “50mM” and “25mM”

- Pipette 1,980µL media into the tube labelled “200mM”

  (this should be done with two pipettes of 990µL)

- Pipette 1,990µL media into the tube labelled “100mM”

  (this should be done with two pipettes of 995µL)

- Pipette 1,995µL media into the tube labelled “50mM”

  (this should be done with two pipettes: one for 1,000µL and one for 995µL)

- Pipette 1,997.5µL media into the tube labelled “25mM”

  (this should be done with three pipettes: one for 1000µL, one for 995µL, and one for 2.5µL)

- Add the ethanol stock into each of the 4 labelled tubes:
  - 200mM – pipette 20µL ethanol stock into the 1,980µL of media
  - 100mM – pipette 10µL ethanol stock into the 1,990µL of media
  - 50mM – pipette 5µL ethanol stock into the 1,995µL of media
  - 25mM – pipette 2.5µL ethanol stock into the 1,997.5µL of media
- Remove the plates from the incubator
- Check the cells under the microscope
- Place inside the safety cabinet (do not spray in as this could kill the cells)
- Open the plates and remove the old media from each well using a multi-channel pipette
- Dispose of the old media in the waste bottle.
- Pipette 100µL of each concentration of media stock into the wells (as per template)
  - 3 wells at 25mM per plate

  - 3 wells at 50mM per plate
  - 3 wells at 100mM per plate
  - 3 wells at 200mM per plate

- Pipette 3 wells per plate with 100µL media and 1µL solvent, but no CLA, to act as a carrier control
- Pipette 3 wells per plate with media only but no cells, and no CLA, to act as a background control
- Return the lid to the well plates and label “0 hours”, “24 hours”, “48 hours” and “72 hours”
- Incubate each plate for the desired time period
- After the desired time, prior to removing the plate from the incubator, thaw the MTS reagent:
  - Remove from freezer
  - Spray into incubator (will take approximately 30min to defrost)
- Remove plate from incubator and place in hood
- Remove the plate lid
- Add 20µL/100µL MTS reagent to each well, using a single pipette
- Cover with lid and incubate again for 2 hours
- Remove the plate from the incubator, and read using a plate reader at 490nm