CHAPTER 6

Study of the regulatory effects on acute apoptosis, cell proliferation and colonic O^6medG by dietary fish oil

6.1. Dietary intervention study

6.1.1. Aims

The following study explores the potential chemopreventative properties of the experimental diets outlined in chapter 5. Diets containing free and encapsulated tuna oil were tested for their ability to regulate a variety of acute endpoints in the rat AOM model. These endpoints are indicative of colorectal cancer initiation in the colon and include the measurement of O^6medG adduct formation, apoptosis and cell proliferation.

The aim of this particular study is as follows;

1. To measure the effect of free tuna oil and encapsulated tuna oil at a variety of doses in the diet on the acute apoptotic response, cell proliferation rates and the O^6medG DNA damage load in the distal colon of rat.

2. To determine whether free tuna oil or encapsulated tuna oil regulate any of these acute host responses in the rat- AOM model to suggest possible protective properties against the early initiation stages of colorectal oncogenesis.

6.1.2. Experimental Rationale

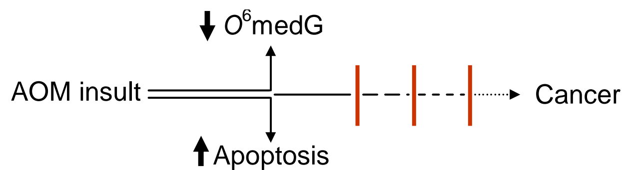
This chapter explores questions that are central to this thesis. It brings together the concept of using both fish oil and the microencapsulated fish oil product as dietary regulators of the acute responses to AOM such as O^6medG and apoptosis. Therefore, not only is the concept of using fish oil as a chemopreventative agent

explored, but the mechanism of this potential protective effect is also investigated.

The modulation of both the acute apoptotic response and O^6medG formation is recognised as having an affect on the eventual development of colorectal cancer. The time course experiment in chapter 4 of this thesis has demonstrated that the formation of O^6medG DNA damage and the acute apoptotic response peak 6h after an insult of carcinogen.

If the O^6medG load is decreased or the apoptotic response is enhanced at this time, the overall level of damage that contributes to mutational events and then potential cancer initiation can be significantly reduced (figure 51). Therefore, any dietary agent that achieves this type of modulation can be considered to have protective properties against the early initiation stages of carcinogenesis.

Figure 51: Modulation of AARGC and O^6medG contributing to a reduction in CRC



The incidence of CRC in the rat can be effectively reduced by agents that either decrease the formation of O^6medG DNA damage or increase the AARGC

Though various long term cancer studies have implied that a diet containing fish oil does have protective properties against colorectal cancer as described in 1.4.5.2, studies carried out using an *in vivo* model and measuring such acute responses to a carcinogen are minimal.

A lone study by Hong *et al.* suggests that a diet of 15% fish oil is protective against colonic tumourigenesis as a result of both increasing the AARGC in the top third of the colonic crypt and reducing the O^6medG load following an AOM insult [69].

This thesis chapter aims to provide further evidence for or against this idea by testing a range of tuna oil diets and microencapsulated tuna oil diets on these same acute endpoints.

AARGC, cell proliferation and O^6medG data were all gathered using small sections of the distal colon taken from experimental animals as outlined in chapter 5. Hence, the reason for choosing the specific dietary regime of each group is explained in 5.1.2.

We hypothesised that a tuna oil diet high in omega-3 fatty acids would reduce the O^6medG level in the distal colon of rats and increase the apoptotic rate. If this were to be the case, this would support the idea that the reported chemopreventative effect of fish oil may be the result of its early modulation of O^6medG DNA damage and removal in the colon. Furthermore, the range of fish oil doses being trialled would allow us to take this investigation to another level and hopefully allow us to establish an optimal fish oil dosage that corresponds to any maximal protective effects observed.

Initially it was also hypothesised that any possible protective effect observed in the fish oil groups may be enhanced in groups fed the microencapsulated fish oil product. This thought initially evolved from the hypothesis that the ME product may deliver fish oil directly to the colon and increase the omega-3 content in the phospholipid of the colon. Though this theory was not supported, as shown in 5.1.4.5, these acute endpoints were still measured in these groups fed the microencapsulated tuna oil product as modulation of these responses are still feasible through other unforseen mechanisms.

The effect of fish oil on the repair protein, MGMT, was also of interest. As any measured increase of this enzyme level could also be related to a possible protective effect against CRC. However, this analysis was not possible with the available tissues. All tissue samples collected from the dietary intervention study were taken 6h post AOM administration. Data suggests that the MGMT enzyme is completely depleted in rat tissues following an insult of an alkylating agent, only appearing once again in tissues after 48h post AOM administration (see

section 1.3.3.2). This was confirmed by analysis of MGMT enzyme using a 32P mitrotitre oligonucleotide assay on colonic samples from rats killed 6h after the AOM insult (performed by PICR). No traces of the MGMT enzyme were detected in any samples tested and hence, analysis of this repair protein was not included among the endpoints.

6.1.3. Study Design

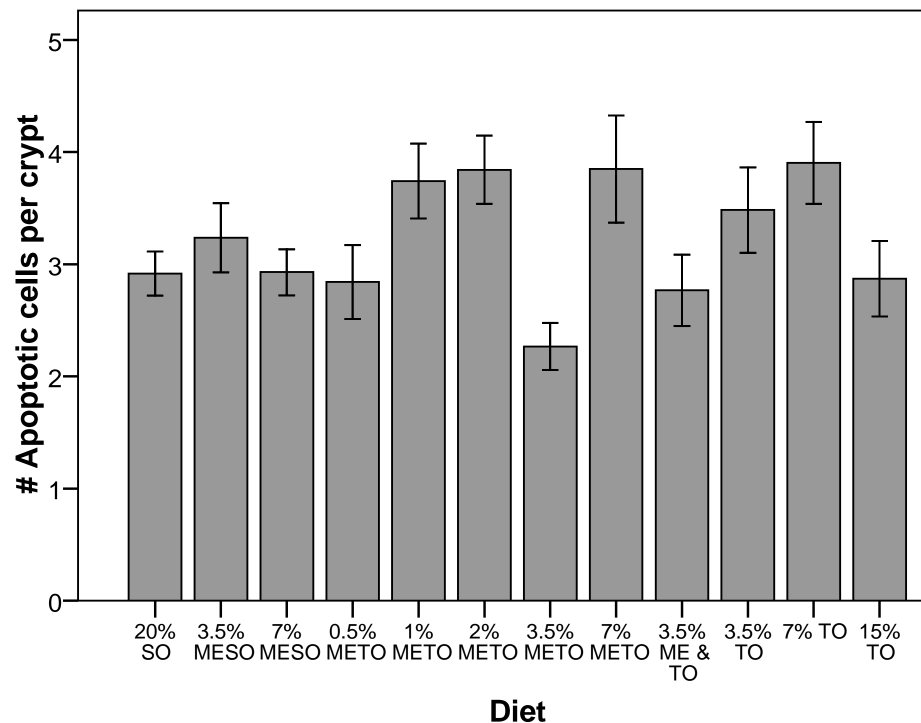
This study used tissues collected from the dietary encapsulation study, therefore, for detailed experimental conditions, diet and protocol please refer to 5.1.3. A small section of distal colon from each animal was removed at the time of kill. These tissue samples were immediately fixed in 10% buffered formalin overnight, processed through an ethanol and xylene gradient and then embedded in paraffin. Tissue sections were cut using a microtome at a thickness of 4μm and stained with the appropriate protocol for the analysis of either apoptosis, cell proliferation or O^6medG DNA adduct formation.

6.1.4. Results

6.1.4.1. Dietary effect on apoptosis

No clear trend was observed between the experimental groups and there were no significant differences found for any group when compared to the 20% SO control which measured an apoptotic count of 2.91 ± 0.19 (SEM) as shown below in figure 52. The highest apoptotic count at 3.9 ± 0.36 (SEM) was measured in the 7% TO group and this was closely followed by the 7% METO, 2% METO and the 1% METO groups at 3.84 ± 0.47, 3.84 ± 0.30 and 3.74 ± 0.33 (SEM) respectively. The lowest count measured was 2.26 ± 0.20 (SEM) in the 3.5% METO group. Inconsistencies in the apoptotic counts between groups suggest that the experimental diets, containing either fish oil alone or microencapsulated fish oil did not have any impact on the total number of apoptotic cells per crypt in response to the AOM insult.

Figure 52: Number of apoptotic cells per crypt

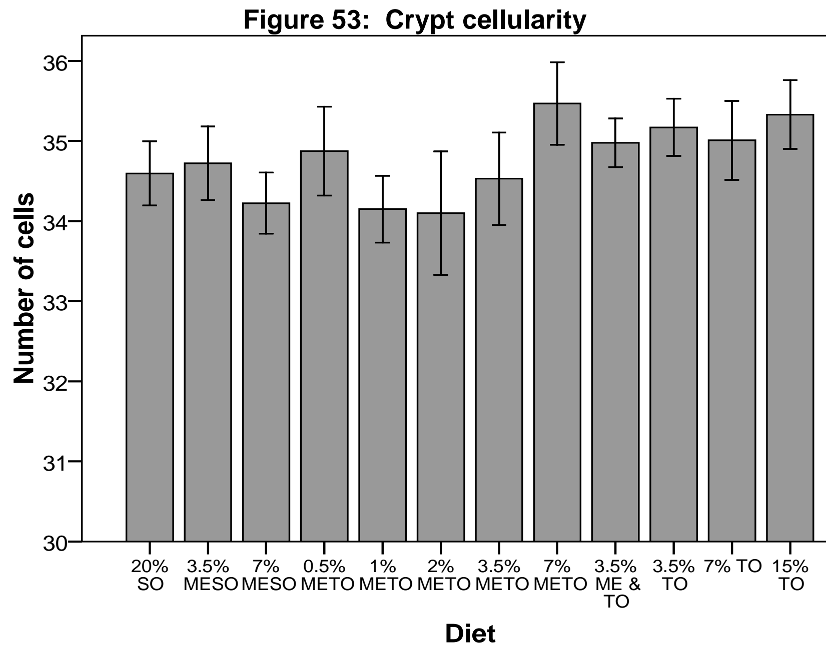


No significant differences were found between any of the dietary groups when compared to the 20% SO control group. Data are means ± SEM for 144 rats (n=12 rats per group).

6.1.4.2. Dietary effect on crypt cellularity and the apoptotic index

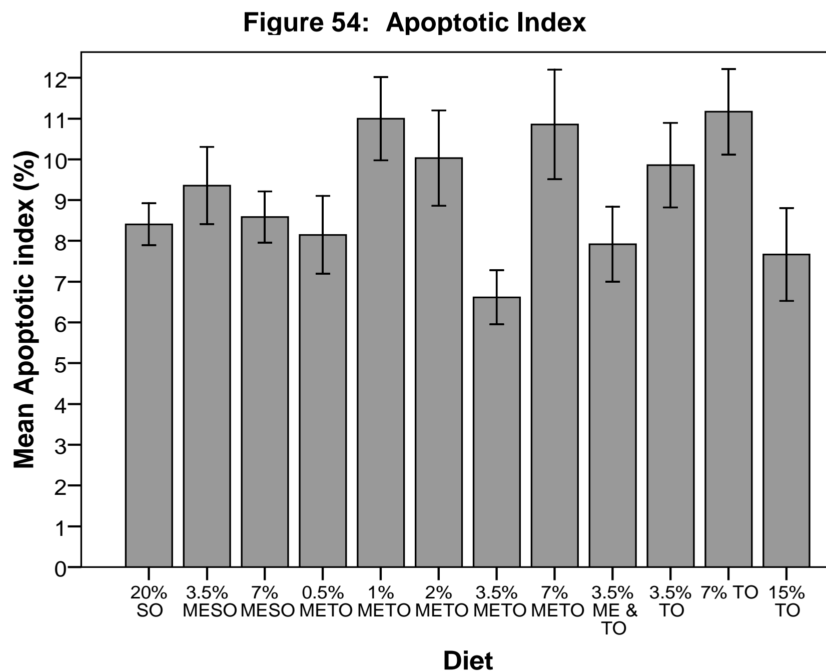
No trends or significant differences were observed in crypt cellularity between groups (figure 53). Counts from all groups fell within a close range of 34.1 and 35.4 cells per crypt. As a result the apoptotic index (% apoptotic cells per crypt) for all groups were comparable to the number of apoptotic cells counted in each crypt (figure 54).

Figure 53: Crypt cellularity



No significant differences were found between groups. Data are means ± SEM for 144 rats (n=12 rats per group).

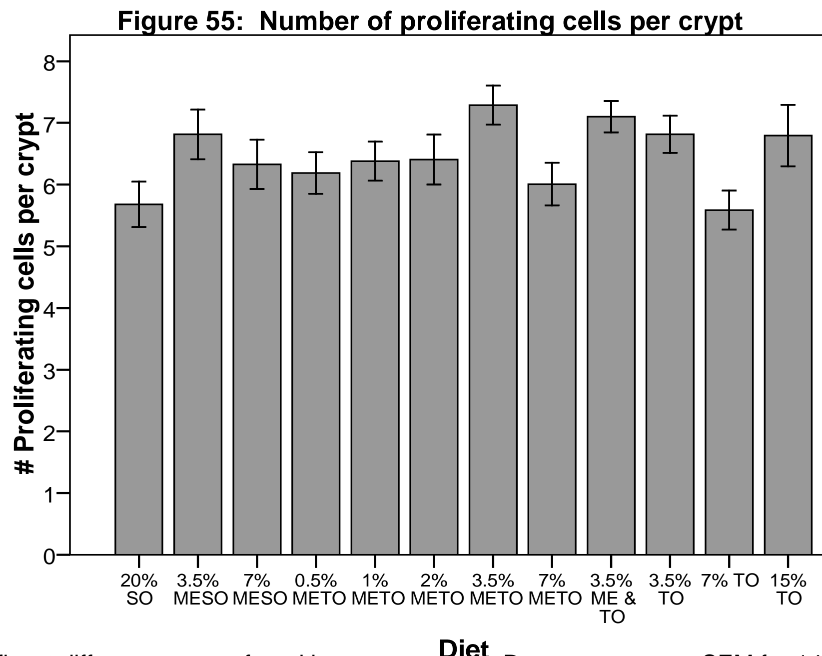
Figure 54: Apoptotic Index



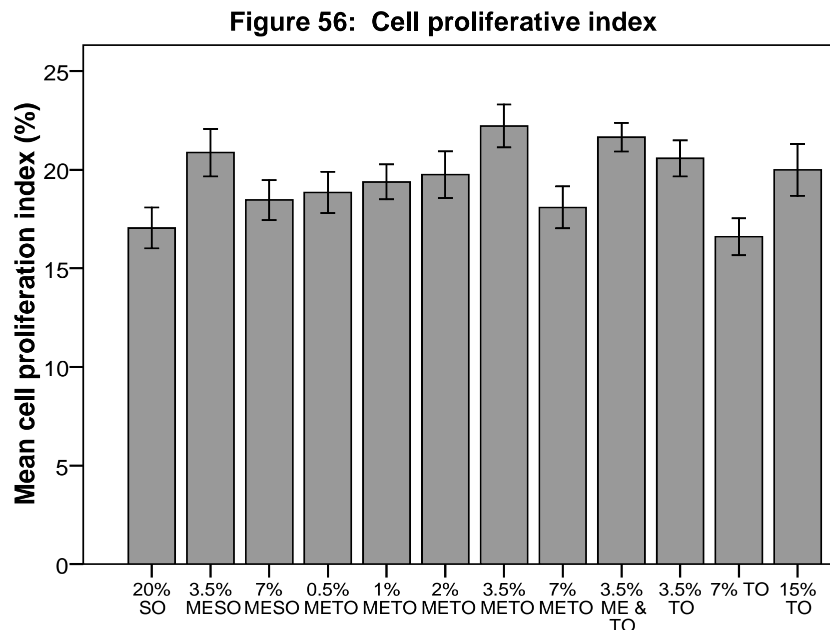
No significant differences were found between groups. Data are means ± SEM for 144 rats (n=12 rats per group).

6.1.4.3. Dietary effect on cell proliferation and the proliferative index

Cell proliferation rates were measured using ki-67. The control 20% SO group had 5.67 ± 0.36 (SEM) or 17.04% of actively proliferating cells per crypt. Again, no trend or significant differences were observed between groups when compared to the control as shown below in figure 54 (number of proliferating cells per crypt) and figure 55 (percentage of proliferating cells per crypt).



No significant differences were found between groups. Data are means ± SEM for 144 rats (n=12 rats per group).



No significant differences were found between groups. Data are means ± SEM for 144 rats (n=12 rats per group).

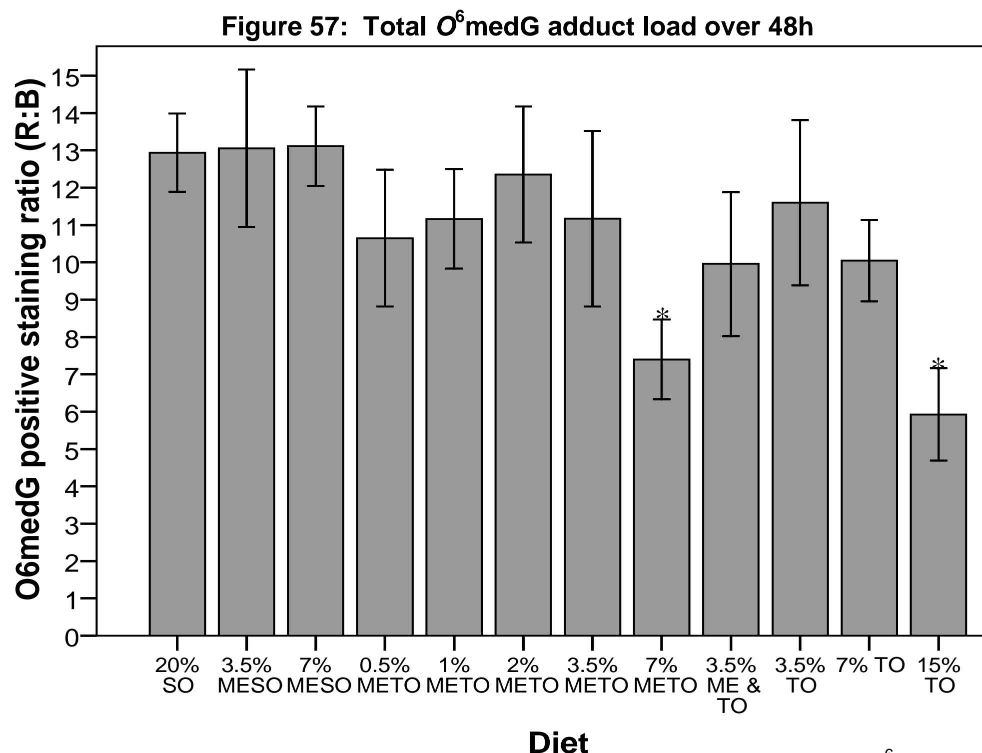
6.1.4.4. Dietary effect on O^6medG DNA adduct

The sunflower oil control diets regardless of dose or form all measured similar levels of the O^6medG DNA adduct, being 12.93 ± 1.1, 13.05 ± 2.1 and 13.1 ± 1.0 (SEM) for 20% SO, 3.5% MESO and 7% MESO respectively. All remaining groups fed on the various fish oil diets had lower levels of O^6medG than all sunflower oil controls.

There was no dose-response relationship or significant differences observed with regard to the lower dose microencapsulated fish oil groups ranging from 0.5% to 3.5% tuna oil. The 3.5% TO group and its equivalent 3.5% METO and 3.5% ME & TO groups were similar in adduct load measuring 11.59 ± 1.1, 11.16 ± 2.3 and 9.95 ± 1.9 (SEM). The 7% TO group also had a comparable level at 10.04 ± 1.0 (SEM).

The 7% METO and the 15% TO diets recorded significantly lower levels (p=0.01 and p=0.003) of O^6medG damage in the colonic epithelium at 7.39 ± 1.0 and 5.92 ± 1.2 (SEM) (see figure 57).

Figure 57: Total O^6medG adduct load over 48h



The 7% encapsulated fish oil and 15% fish oil groups had significantly reduced O^6medG adduct loads in the distal colon when compared to the 20% sunflower oil control diet * p<0.01 (ANOVA, Tukey). Data are means ± SEM for 144 rats (n=12 rats per group).

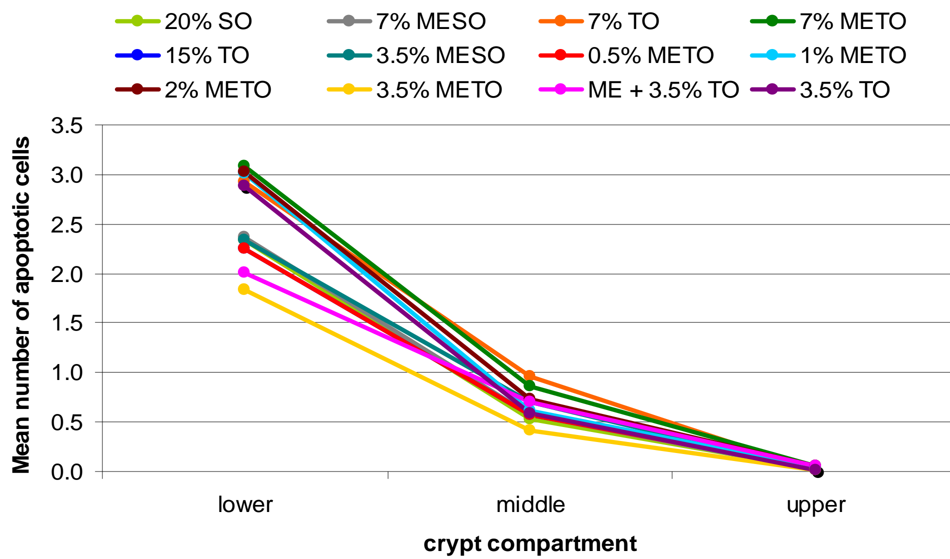
6.1.4.5. Dietary effects on the distribution of apoptosis, cell proliferation and O^6medG throughout the colonic crypts.

As in section 4.1.4.9, the distribution of the measured host responses for each dietary group was analysed along the length of the crypt. This data is summarised in the following three graphs for the apoptotic response, the cell proliferation rate and the average O^6medG load in each of the lower, middle and upper crypt thirds.

As shown in chapter 4, the apoptotic response primarily occurred to epithelial cells in the lower third of the colonic crypts (figure 58). The change in total apoptosis rates, while not significant, did seem to reflect a change in the rate of apoptosis in the lower third of the crypt as opposed to the other two zones. While no notable difference was observed between the dietary groups with respect to the distribution of the number of proliferating cells along the crypt length (figure 59).

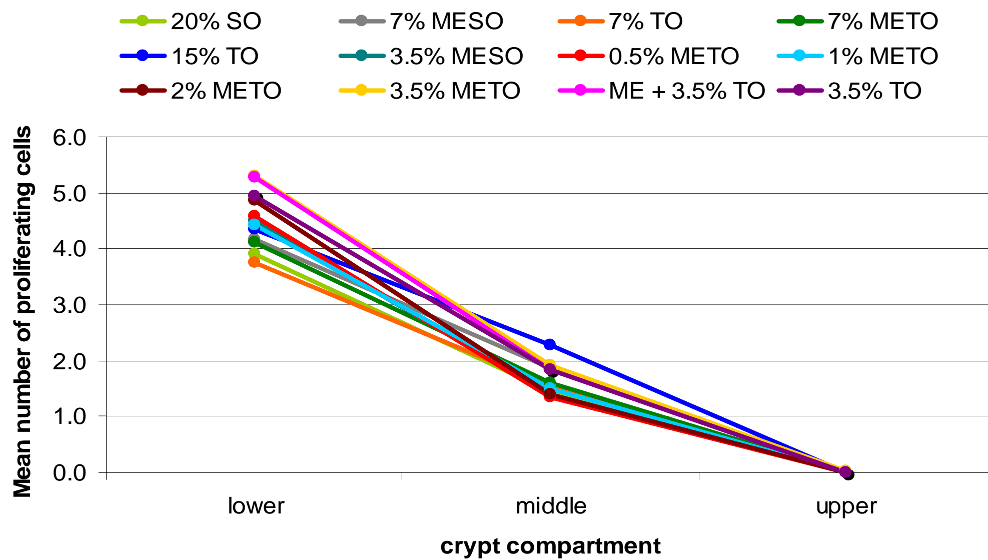
The general pattern of O^6medG levels in the three compartments, as shown in figure 60, is similar for each dietary group, with O^6medG levels decreasing from the lower to the upper compartments. This graph also shows us that 7% METO and the 15% TO groups which had a significantly lower total O^6medG loads, were lower than all other groups in both the lower and middle compartments, with no obvious change being observed in the upper crypt compartment.

Figure 58: Effect of diet on the apoptotic response by crypt compartment



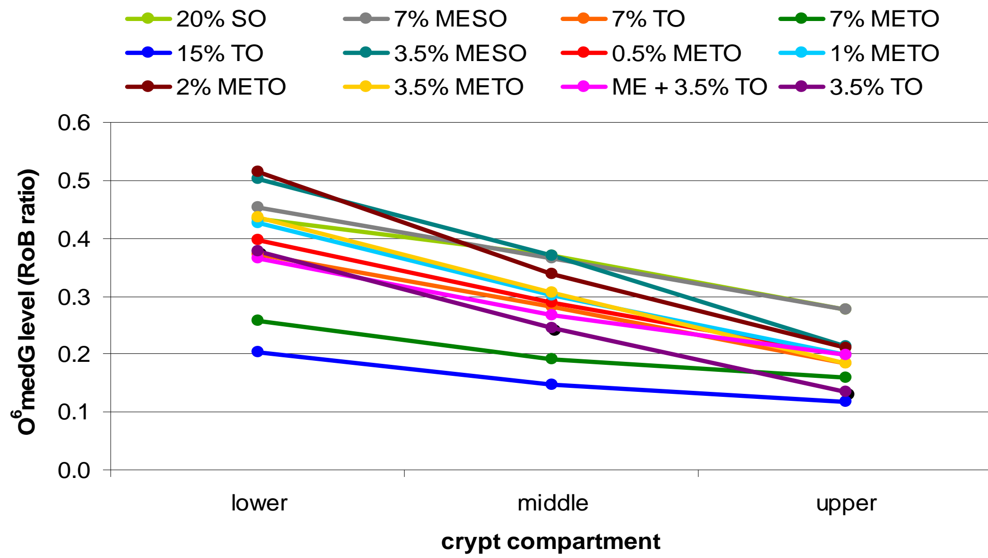
This graph displays the mean number of apoptotic cells per crypt for each of the 3 compartments of a colonic crypt for each dietary group. 'Lower' represents the mean count for cell positions 1-12, 'middle' represents the mean count for cell positions 13-24 and 'upper' represents the mean count for cell positions 25-37.

Figure 59: Effect of diet on cell proliferation over time by crypt compartment



The graph above displays the mean number of proliferating cells per crypt for each of the 3 compartments of a colonic crypt for each dietary group. 'Lower' represents the mean count for cell positions 1-12, 'middle' represents the mean count for cell positions 13-24 and 'upper' represents the mean count for cell positions 25-37.

Figure 60: Effect of diet on *O*^{6}medG load over time by crypt compartment



The graph above displays the mean O^{6}medG load per crypt for each of the 3 compartments of a colonic crypt for each dietary group. 'Lower' represents the mean count for cell positions 1-12, 'middle' represents the mean count for cell positions 13-24 and 'upper' represents the mean count for cell positions 25-37.

6.1.5. Discussion

This particular study was designed to; 1) investigate the affects of tuna oil on early biomarkers of cancer initiation and 2) investigate the affects of microencapsulated tuna oil on early biomarkers of cancer initiation. This was completed by measuring the AARGC, O^6medG load and cell proliferation rates in the rat-AOM model.

A study carried out by Hong *et al*. has previously shown modulation of these biomarkers using a high fish oil diet [69]. Given this information and the reported anti-tumourigenesis properties that fish oil has, we hypothesised that tuna oil in the diet will reduce the O^6medG load and increase apoptosis in the colon.

The modulation of apoptosis by fish oil was not supported by our findings. Fish oil either free or encapsulated in the diet did not increase the number of apoptotic cells in the colon. Additionally, no clear trend was noted with regard to the different doses of fish oil and the level of apoptosis measured.

The modulation of O^6medG was supported however by a high fish oil diet. Animals fed a 15% tuna oil diet had significantly lower levels of colonic O^6medG across both the lower and middle compartments of the crypts. The mechanism by which tuna oil helps to reduce colonic O^6medG remains unclear, though one would suggest as a result of the distribution data that the mechanism appears to be affecting cells within the bottom two thirds of the colonic crypt.

A number of mechanisms may be attributed to this inhibitory effect of O^6medG by tuna oil. Firstly, it is possible that the lower levels of O^6medG in colonic cells are the result of the repair of damage via MGMT or the removal via apoptosis in particular. However, the fact that either total apoptosis or apoptosis in the lower third of the crypt did not increase in response to the addition of tuna oil in the diet suggests that the reduction of O^6medG as a result of apoptosis is unlikely.

It is possible however, that the reduction of O^6medG as seen in animals fed the high fish oil diets may be in part due to an increase the cells capacity to repair this type of damage. The role of the MGMT repair protein is to specifically remove the O^6medG lesion from double stranded DNA. As one MGMT molecule can only remove only one O^6medG lesion, the level of O^6medG repair is dependent on the amount of MGMT present in cells. Fish oil may enhance levels of this enzyme in colonic epithelial cells and therefore the cellular capacity to repair O^6medG may be increased which in turn leads to a lower level of damage.

Studies show that MGMT levels are depleted following an insult of alkylating agent. Our pilot investigations also showed that MGMT levels in colonic tissue 6h post AOM were not detectable, therefore, this repair endpoint could not be measured on the available samples. However, the possibility of MGMT modulation by fish oil is still feasible. Therefore, the study of the effect of a high dose fish oil diet on MGMT levels is recommended. This can be accomplished by either measuring MGMT levels prior to an insult of carcinogen or at a later time point when cellular MGMT levels have recovered.

It is also possible that the higher incorporation of omega-3s into membrane phospholipids resulting from a high tuna oil diet could simply promote the formation of more favourable omega-3 derived eicosanoids that promote bowel health (as discussed in 5.1.4.5). The uptake of omega-3 PUFAs into the phospholipid membrane may also have an affect on wide variety of enzymes, transcriptions factors or genes that may help potentiate this reduction of O^6medG.

Alternatively, if one is to question the effect of omega-3 on the general workings of the cell, the question also must be asked of the potential interplay between omega-3 PUFAs and the AOM carcinogen used in this model. It is possible that the increase in omega-3 PUFAs in tissues can also have an effect on a range of other enzymes and these may include those responsible for the metabolism of the carcinogen used to induce the DNA damage in the beginning. This area is explored further in section 6.2.

Animals fed the 7% microencapsulated tuna oil diet were the only other group to have significantly lower levels of colonic O^6medG. This reduction in total O^6medG levels was not seen in the 7% free tuna oil group. One may presume then, that in this case, the tuna oil when encapsulated is more beneficial in reducing the level of acute DNA damage in the colon.

The reason as to why the microencapsulated oil delivers more promising results is unclear. Long chain fatty acid analysis of all tissues in both the 7% free and encapsulated tuna oil groups shows that the omega-3 levels were equivalent across all samples measured. Therefore, an omega-3 effect on tissue phospholipid is not likely to be the reason for this differential effect between groups.

It may be the case that the method of encapsulating tuna oil is in fact reducing the total level of colonic damage through means other than its potential mode of direct delivery to the colon. As a significant increase in caecal butyrate levels were observed with this group it is feasible that the reduction in O^6medG may have been the result of a synergistic effect of both the 7% tuna oil and the butyrate enhancing outer capsule.

Ideally, the separation of oil and capsule in the diet as done in the 3.5% group would have been of great use if carried out at a dose of 7%. If this was the case, an insight may have been gained into what mechanism may have contributed to this result; the delivery of the oil or simply the combination of the dietary oil and capsule components?

Following on from this, it could also be assumed that an increase in apoptosis should also be seen animals fed this 7% microencapsulated diet. Butyrate is more predominately recognised for its pro-apoptotic qualities [77] and therefore, if levels were significantly high enough to contribute to the reduction of colonic O^6medG, in theory the number of apoptotic cells per crypt should have also increased. This is supported by *in vitro* studies that show that a combination of both DHA and purified sodium butyrate potentiate this apoptotic effect in cells [119, 150]. However, significant changes were not observed in any of the

encapsulated tuna oil groups with regard to apoptosis and therefore this synergistic effect was not supported by our findings.

6.2. N7meG study

6.2.1. Aims

The objective of the following additional study was to determine whether a diet high in omega-3 PUFAs can influence the metabolism of AOM in the *in vivo* model of colorectal carcinogenesis. While it was hypothesised that omega-3 PUFAs would not interfere with the metabolism of AOM in the rat, this study was needed in order to help determine whether the reduction in O^6medG, as observed in animals fed the high dose tuna oil diet, was a true and direct effect of the experimental diet and not the result of any dietary interference with the rat-AOM model itself.

The specific aims of this particular study include;

1. To measure the N7meG DNA adduct load in the distal colon of AOM injected rats fed a control diet and compare these to N7meG levels in rats fed a 15% tuna oil diet.

2. To then relate levels of N7meG DNA damage to levels of O^6medG DNA damage and establish whether a diet high in tuna oil may affect the metabolism of AOM *in vivo*.

6.2.2. Experimental Rationale

Data from the previous acute dietary intervention study showed that a diet containing either 7% microencapsulated tuna oil, or 15% free tuna oil, can lower the total O^6medG DNA adduct load in the distal colon of rats. As the amount of acute O^6medG damage in the colon has been shown to be indicative of eventual tumour formation [62, 66], these findings imply that high dose tuna oil may also have chemopreventative properties against the formation of CRC.

While this mechanism by which omega-3 PUFAs act to reduce the O^6medG load still remains unclear, it is important to validate our findings as a direct effect of

omega-3 on O^6medG formation rather than a potential indirect interference on the metabolism of the carcinogen used.

The majority of chemical carcinogens must be metabolised by a family of P450 enzymes to reactive electrophiles in order to react with DNA and initiate a carcinogenic response. Therefore, the relative amounts of these enzymes play an important role in determining the potency of a carcinogen [198]. It has been shown that the uptake of omega-3 PUFAs into liver tissue as a result of consuming fish oil influences these types of drug activating enzymes in rats. Yao and colleagues [199] demonstrated the inhibitory effects of both EPA and DHA on a series of CYP enzymes involved in drug metabolism, including CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4.

Of particular interest is cytochrome P450 2E1, otherwise known as chloroxanzone 6-hyroxylation. This CYP enzyme has been shown to play a significant role in the metabolism of AOM by catalysing the oxidative conversion of AOM into the ultimate chemical carcinogen in both the liver and colon of rats [157].

The importance of this enzyme in the process of AOM activation and its ability to affect the workings of the rat-AOM model has been supported by work carried out by Sohn and colleagues [200]. Using a CYP2E1-null mice model, AOM was administered to these null mice and a wild type control group. Results clearly verified that a CYP2E1 deficiency can affect the activation of AOM in rats as reflected in the significant reduction of acute colonic O^6medG by 87% and also long term ACF formation by 70% in the CYP2E1 null mice. Additionally, agents deemed to have chemopreventative properties such as disulfiram have since been identified as inhibitors of CYP2E1 [201].

Knowing that omega-3 PUFAs may have an inhibitory effect on CYP2E1 and that the inhibition of this enzyme can result in the interference of AOM metabolism, it is then important to ask the question; Was the reduction of O^6medG observed in animals fed 15% tuna oil the result of an inhibitory effect on the metabolism the carcinogen AOM?

To test this, it was decided that another DNA adduct, known as N7meG, which is also formed as a result of AOM administration would be measured in the liver tissue of selected rats from the previous dietary intervention study.

N7meG is an alkylating DNA adduct which is formed as a result of a methyl group attaching to the nitrogen at position 7 in a guanosine base in DNA. N7meG is the most readily formed adduct when DNA is subjected to an alkylating agent however, unlike O^6medG, the N7meG adduct has no carcinogenic or mutational properties [54]. It is also repaired at an extremely low rate and it can therefore, be a good indicator of the approximate tissue exposure to a carcinogenic insult, in this case the AOM insult.

As a result, liver N7meG levels from selected dietary groups were analysed. With this information, it can then be established whether the metabolism of AOM in the rat model is inhibited by the addition of omega-3 in the diet. And furthermore, whether the reduction of O^6medG DNA damage as observed in the 15% tuna oil diet is in fact an accurate chemopreventative effect and not simply a product of an altered AOM metabolism.

6.2.3. Study Design

The N7meG levels of 3 groups were analysed. Firstly, a control group fed the standard 20% sunflower diet and administered with saline was tested to establish a baseline level of N7meG in the tissue of untreated rats. N7meG levels were then measured in rats administered with 10mg/kg b.w. AOM and fed the standard 20% sunflower oil control diet and also the 15% tuna oil diet.

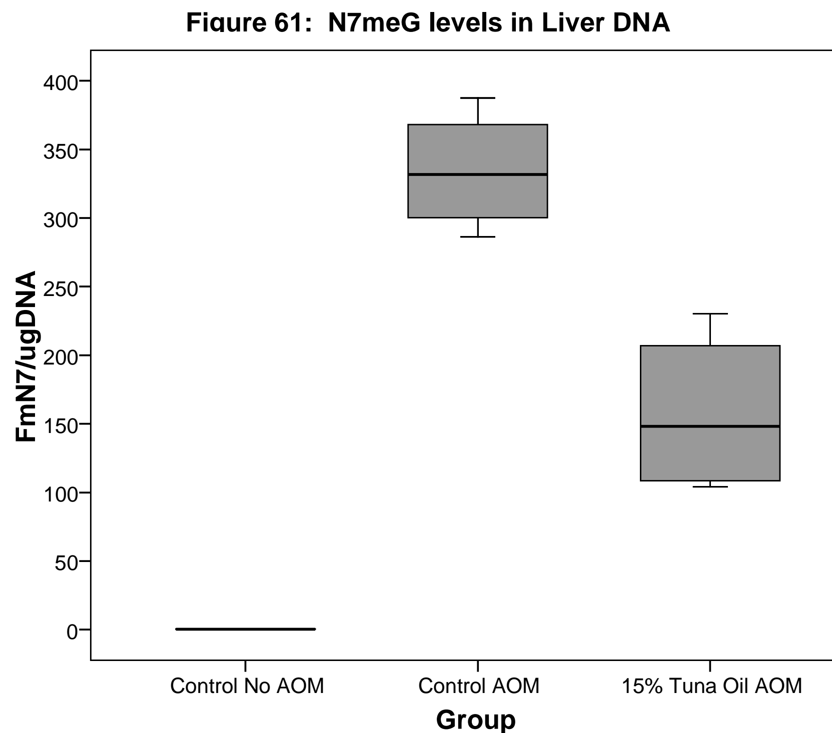
The liver tissue from 4 randomly selected rats from each group were prepared and assayed for N7meG levels using an immunoslotblot assay at PICR. It was decided that liver tissue would be analysed, as though a degree of local activation of AOM is believed to occur in colonic epithelial cells, the liver contains significant amounts of CYP 2E1 and is the primary organ at which the oxidation of AOM occurs [202].

6.2.4. Results

6.2.4.1. N7meG DNA adduct levels

Control animals that were administered a saline injected recorded baseline levels of 0.46 ± 0.1 (SEM) of the N7 DNA adduct as shown in figure 58 and table 19. Animals injected with 10mg/kg b.w. AOM and on the 20% control sunflower oil diet had the highest level of N7meG formation in the liver at 334.21 ± 21.8 (SEM). While rats fed the 15% tuna oil diet had significantly lower (p=0.003) N7meG formation in DNA at 157.72 ± 30.0 (SEM).

Figure 61: N7meG levels in Liver DNA



All data expressed as means ± SEM for n=4 rats. The 15% fish oil group had significantly lower levels of N7meG in the liver when compared to the control group (p=0.003).

Table 19: N7meG Levels in Liver DNA

Diet	Treatment	N7/µg DNA	n
Control	Saline	0.46 ± 0.1	4
Control	AOM	334.21 ± 21.8	4
15% Tuna oil	AOM	157.72 ± 30.0	4

Experimental summary for each group including diet, treatment, mean N7meG level and rat number. N7 data expressed as means ± SEM for n=4 rats.

6.2.5. Discussion

As previously shown, animals fed a diet containing 15% tuna oil had significantly reduced O^6medG levels in the colon. The purpose of this additional study was to determine whether this result may have been influenced by an impaired AOM metabolism caused by a diet high in omega-3 PUFAs.

Using the N7meG as a biomarker of AOM activation, this DNA adduct was measured in the liver tissue of animals on both a control diet and a 15% tuna oil diet. A significant reduction of N7medG levels were observed in the liver tissue of animals fed a 15% tuna oil diet.

If one accepts the N7medG adduct as an accurate marker of the quantity of AOM that has been catalysed into the final active carcinogen, these findings suggest that animals fed a diet of 15% tuna oil were not subjected to the same dose of carcinogen as animals which were fed a control diet. Moreover, it can then be implied that a diet high in omega-3 PUFA interferes with the metabolism of AOM. This interference may possibly occur as a result of the inhibition of the enzyme CYP 450 2E1 [199].

With this said, the O^6medG data from the various tuna oil fed groups must then be cautiously interpreted. An accurate comparison between the endpoints measured in these groups can not necessarily be made if the exact dose of final carcinogen delivered to each animal was interfered with and therefore unknown.

The different N7meG levels in both the tuna oil and control groups imply an altered AOM metabolism. Yet it is important to understand that further work is needed to confirm these results and an array of possibilities have yet to be examined. It has been established that AOM is primarily metabolised in the liver [158]. However, analysing the N7meG load in the colonic tissue of these animals would also be of great value. Especially when considering that the endpoints measured in this study pertain to an AOM insult specifically on colonic tissue.

Not only can colonic tissue be analysed for N7meG levels, but tissue samples from the various tuna oil fed animals could also be analysed. If a dose-response relationship was found between the N7meG values and the amount of omega-3 in the diet or alternatively in the tissue phospholipid membrane, support for the idea that omega-3 PUFAs do affect AOM metabolism can be strengthened.

However if one was to accept the idea that the N7meG levels represent the amount of carcinogen metabolised, one pertinent question remains. Why then, was this effect not also represented in the apoptotic data?

While no information could be found regarding the effect of different doses of carcinogen on the AARGC response, one may assume that if a lower level of carcinogen was metabolised in the high dose tuna oil fed groups, then the apoptosis rates in these groups may also be lower. However, this was not the case, with the number of apoptotic cells per crypt being similar in both the 20% sunflower oil control group and the 15% tuna oil group (as shown in 6.1.4.1). An extended study observing the effects of different doses of AOM on the AARGC may be helpful in exploring this issue further.

It may also be the case that a diet high in omega-3 PUFAs simply enhances the function or metabolism of repair proteins specific to N7meG. It is accepted that the N7meG lesions repair capability is minimal and extremely slow in tissues [54]. However, it is possible that a diet containing a high dose of fish oil may activate or induce repair enzymes to such an extent that the lower N7meG level is not indicative of AOM metabolism but rather an increased repair capacity.

This experiment still poses a number of questions and perhaps the method best suited to investigate this further involves the measurement of the AOM activating enzymes themselves. By assaying the CYP2E1 enzyme in both the liver and colon of rats, one does no have to rely on inferred information and any influence of omega-3 PUFAs on the metabolism of AOM can be analysed. The measurement of CYP2E1 by immunoassay has been previously outlined [203] and conducting this assay on the samples generated from this dietary intervention study would be my primary recommendation for taking this hypothesis further.