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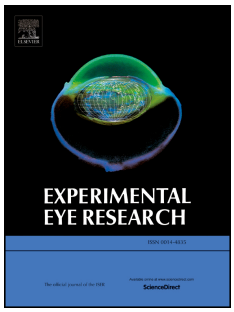

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Lutein and Zeaxanthin attenuates VEGF-induced neovascularisation in human retinal microvascular endothelial cells through a Nox4-dependent pathway

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Abstract

Age-related macular degeneration (AMD) and proliferative diabetic retinopathy (DR) are two of the most common and severe causes of vision loss in the population. Both conditions are associated with excessive levels of vascular endothelial growth factor (VEGF) in the eye which results in an increase in the formation of new blood vessels through a process called neovascularisation. As such, anti-VEGF therapies are currently utilised as a treatment for patients with AMD however they are associated with painful administration of injections and potential degeneration of healthy endothelium. There is therefore growing interest in alternate treatment options to reduce neovascularisation in the eye. The use of carotenoids, lutein (L) and zeaxanthin (Z), has been shown to improve vision loss parameters in patients with AMD, however the underlying mechanisms are not well-understood. We studied the impact of these compounds on neovascularisation processes using an *in vitro* cell model of the retinal microvascular endothelium. Our findings show that L and Z reduced VEGF-induced tube formation whilst, in combination (5:1 ratio), the compounds significantly blocked VEGF-induced neovascularisation. The carotenoids, individually and in combination, reduced VEGF-induced oxidative stress concomitant with increased activity of the NADPH oxidase, Nox4. We further demonstrated that the Nox4 inhibitor, GLX7013114, attenuated the protective effect of L and Z. Taken together, these findings indicate the protective effect of the carotenoids, L and Z, in reducing VEGF-mediated neovascularisation via a Nox4-dependent pathway. These studies implicate the potential for these compounds to be used as a therapeutic approach for patients suffering from AMD and proliferative DR.

Age-related macular degeneration (AMD) is a leading cause of vision loss in the ageing population (Bourne et al. 2014). The pathogenesis of AMD is multifactorial with a variety of associated factors including age, genetic contribution and environmental stress. The clinical hallmark of dry AMD is the appearance of localised deposits of oxidised lipids and proteins within the eye, known as drusen, which form between the basement membrane and Bruch's membrane (Gorusupudi et al. 2017). The most devastating hallmark of AMD is seen in patients with the wet-form of the disease in which neovascularisation occurs resulting in sudden and irreversible loss of vision with intra-vitreal injections the treatment option (Ferris et al. 1984).

Severe cases of diabetic retinopathy (DR) can also lead to neovascularisation (in proliferative DR) leading to irreversible loss of vision (He, M. S. et al. 2018, Topouzis et al. 2009). Worldwide, there were an estimated 425 million people suffering with diabetes in 2017 which is projected to reach 629 million in 2045 (Ogurtsova et al. 2017). DR is a serious complication in chronic, poorly-controlled diabetes (National Eye Institute) and a leading cause of vision impairment and loss in the working population (Porta and Bandello. 2002). The prevalence of vision-threatening proliferative DR which can lead to blindness is 6.32% in Europe and 7.26% worldwide (Teo et al. 2020).

DR is a complex, multifactorial disease which progresses from a mild, non-proliferative condition, characterised by breakdown of the retinal microvasculature, pericyte loss and capillary degeneration, to a proliferative form of the disease which is associated with new blood vessel formation (Fong et al. 2003, Wong et al. 2016). This neovascularisation can bleed and cause mechanical traction and result in retinal detachment and subsequent blindness (Stitt et al. 2013). Prolonged hyperglycaemia in diabetic patients results in metabolic disruption within the retina, with the release of a number of growth, neurotrophic and inflammatory factors (Ola et al. 2012, Qian and Ripps. 2011, Tarr et al. 2013). Many of these released molecules result in vascular disruption due to the key pathogenic factor released in DR, vascular endothelial growth factor (VEGF). Hypoxia which is observed in patients with proliferative DR, elevates VEGF levels, to result in increased migration and proliferation of retinal endothelial cells to form new blood vessels (Simo et al. 2006). Hyperglycaemia-induced oxidative stress in the microvasculature is therefore a key pathology related to the development of DR. As such, excessive reactive oxidative stress (ROS) accumulation occurs in the retina of diabetic patients (Calderon et al. 2017, He, M. et al. 2013, Tan, J. S. et al. 2008). Cytosolic NADPH oxidases, Nox, are the primary enzyme family which is responsible for generating cellular ROS by catalysing the reduction of molecular oxygen to the superoxide anion via oxidising NADPH to NADP. Whilst there are three Nox isoforms found in the retina (Nox1, Nox2, Nox4), Nox4 is the predominant isoform (Serrander et al. 2007) and has been closely linked with the development and severity of DR in type 2 diabetic patients (Appukuttan et al. 2018, Ibrahim et al. 2015, Kim et al. 2012, Kowluru et al. 2014, Li et al. 2010, Meng et al. 2018, Wang et al. 2014). Indeed, Nox4 activity is associated with endothelial cell processes linked to retinopathy including permeability, hyper-proliferation and tubulogenesis (Li et al. 2010). The inhibition of Nox4 therefore represents a potential therapeutic mechanism to target various microvascular complications seen in retinopathy.

For proliferative DR, panretinal photocoagulation therapy has been the gold standard for treatment of DR to prevent severe vision loss. The therapy is, however, inherently a destructive therapy resulting in potential loss of photoreceptors, and therefore loss of peripheral field and night vision (Sun, J. K. et al. 2019), as well as vitreal haemorrhage (Coney. 2019). Anti-VEGF treatments, such as the full-length recombinant humanized anti-VEGF monoclonal antibody Bevacizumab (Avastin)

(Ferrara et al. 2004) and the recombinant antibody fragment of humanised anti-VEGF monoclonal antibody Ranibizumab (Lucentis) (Heier et al. 2006), have been recently been advocated as alternative therapies which have been successful in diabetic and AMD clinics. These are administered as intravital injections and can therefore be painful for patients and associated with a range of potential complications including cataract, infectious endophthalmitis, conjunctival haemorrhage and retinal detachment (Coney. 2019).

There is therefore a growing need to understand the development of novel therapeutic agents to inhibit VEGF activity and thus reduce retinal injury and improve vision outcomes for patients with diabetes and AMD. Whilst different vascular beds undergo neovascularisation in each disease, retinal endothelium for DR and choroidal endothelium for AMD, finding therapeutic interventions for DR are also likely to have a positive effect on the incidence of irreversible loss of vision. It is therefore vital to understand the molecular mechanisms which underlie neovascularisation in DR pathology, and use this information to develop therapeutic options for patients.

We have previously identified the presence of novel GPCRs, T1R2/3, in human retinal microvascular endothelial cells, which are activated by a range of acutely sweet molecules (Lizunkova et al. 2019). Our studies demonstrated that the sweetener, sucralose, blocked VEGF-induced angiogenic processes such as permeability and neovascularisation, in the human retinal microvasculature. These studies indicate the potential link between dietary components and improved outcomes for patients with neovascularisation and pave the way for new techniques to tackle the condition. Indeed, recent clinical studies have investigated the potential for a range of dietary components, including micronutrients and vitamins, to reduce various aspects of retinopathy pathology including age-related macular degeneration (Chew et al. 2013, Dow et al. 2018, Kowluru, Kanwar et al. 2008, Lee et al. 2010). Of these, two of the most extensively studied are lutein (L) and zeaxanthin (Z). These xanthophyll carotenoids are endogenously expressed at high levels in the retina however there is no *de novo* synthesis of either therefore dietary consumption of these molecules is important for vision (Scripsema et al. 2015). Indeed, in a range of clinical studies, such as CARMIS, LUTEGA and AREDS2, L and Z at different ratios have been shown to protect against markers of retinopathy in AMD (Chew et al. 2013, Dawczynski et al. 2013, Piermarocchi et al. 2012). In the Blue Mountain Study, supplementation of L and Z is associated with as much as 65% reduced risk of neovascular AMD (Tan, J. S. et al. 2008). There are, however, limited studies which assess L and Z supplementation in DR or the molecular mechanisms through which these micronutrients impact the endothelium. *In vivo* studies indicated that L and Z act as antioxidants to preserve retinal function and prevent neuronal loss in the retina of diabetic models (Arnal et al. 2009, Kowluru, Menon et al. 2008, Miranda et al. 2006, Sasaki et al. 2010). In humans, Hu *et al* demonstrated that L and Z supplementation in patients with non-proliferative DR resulting in improved visual function as assessed by visual acuity, contrast sensitivity and macular pigment optical density (Hu et al. 2011). Whilst L and Z are found in different foods such as spinach, corn and eggs, many studies have tested commercially-available supplements, including Macushield which comprises of a 5:1 ratio of L to Z, in patients with AMD (Akuffo et al. 2015, Al-Ahmary. 2010, Crosby-Nwaobi et al. 2016). Despite these studies, there is limited understanding of the effect of L and Z at a cellular level in the retina and specifically how these compounds could be efficacious in reducing retinal neovascularisation, associated with DR.

We have therefore sought to address this using physiologically-relevant concentrations of L and Z, or combination treatment (5:1 lut:zeax), in human retinal microvascular endothelial cells. We utilised

VEGF as an *in vitro* model of DR and the anti-VEGF Bevacizumab as a positive control for currently available pharmaceutical therapy.

Human retinal microvascular endothelial cells (RMVEC) were purchased from Cell Systems (Kirkland, WA) and cultured specialised supplemented vascular cell media using culture boost. Cells were utilised between passages 2 and 7 only and routinely checked for endothelial cell characteristics including vascular endothelial cadherin expression and uptake of acetylated LDL. Unless otherwise stated, all reagents were sourced from Sigma-Aldrich.

Cell viability was assessed using 3-4,5-dimethylthiazole-2-yl-2,5-diphenyltetrazolium bromide (MTT). RMVEC were exposed to bevacizumab, L, Z or 5:1 lut:zeax, or vehicle (H_2O), treatment at a range of concentrations (0.05, 0.075. 0.1, 0.25, 0.5, 1 µg/µl) for 24 h, followed by incubation with MTT reagent for 2 h at 37^{o}C. Absorbance was assessed at 570 nm using a microplate reader (Victor Perkin-Elmer) and viability was calculated as % normalised to vehicle. Cellular ROS was assessed using the fluorogenic dye 2',7'–dichlorofluorescin diacetate (DCFDA). RMVEC were exposed to DCFDA (10 µM) for 30 min at 37^{o}C and then replaced with bevacizumab, L, Z, or 5:1 lut:zeax with VEGF (50 ng/ml) or vehicle (H_2O). DCFDA fluorescence was measured at 488 nm using a fluorescent plate reader (Victor, Perkin Elmer) for time points from 10 to 240 mins.

Cellular glutathione (GSH) and Nox4 activity were quantified using commercially-available kits. For all kits, RMVEC were plated in a 96-well plate and exposed to bevacizumab, L, Z or 5:1 lut:zeax in the presence and absence of VEGF (50 ng/ml), for 24 h. For the GSH Bioxytech activity kit (Merck Millipore, Hertfordshire, UK), levels of the monochlorobimane dye bound to reduced or oxidised glutathione were quantified by fluorescence measured at an excitation and emission of 380/461nm using a fluorescent plate reader (Victor, Perkin Elmer). For the Nox4 activity kit (Cusabio, Texas, USA), the assay was performed as per the manufacturer's protocol and absorbance was measured at 450nm using a microplate reader (Tecan Sunrise).

Angiogenic processes were measured as cell proliferation, migration, adhesion and neovascularisation as previously described (Lizunkova et al. 2019). In brief, RMVEC were quiesced for 24 h with 1% FBS followed by exposure to bevacizumab, L, Z or combined treatment, in the presence and absence of VEGF, for 6 h. Alternatively, cells were exposed to bevacizumab, L, Z or combined treatment, with VEGF (50 ng/ml), in the presence of GLX7013114 (1 µM) (Raystar Bio, Hangzhou Guangyuan, China) or the vehicle control (DMSO). To ensure that bevacizumab did not neutralise VEGF in preparation, VEGF treatment was administered separately but within a 5 minute window. Cells were then counted using a haemocytometer for the cell proliferation assay. For cell migration assay, RMVEC were plated for 24 h, followed by a scratch using a pipette tip and immediately expose to bevacizumab, L, Z or 5:1 lut:zeax, in the presence and absence of VEGF (50 ng/ml). Migration was then monitored at 2 h time intervals and images were captured at × 10 magnification using a Zoe™ Cell Imager (BioRad). Cell migration was assessed using the MiToBo analyser software in Image J as previously described (Lizunkova et al. 2019). Cell adhesion was assessed following exposure to bevacizumab, L, Z or 5:1 lut:zeax, in the presence and absence of VEGF (50 ng/ml), for 2 h. RMVEC were then rinsed and MTT assay was performed as for the viability assay to quantify adhered cells. Neovascularisation was measured by plating cells onto Matrigel™-coated plasticware (BD Biosciences, Oxford, UK) and immediately exposing cells to bevacizumab, L, Z or 5:1 lut:zeax, in the presence and absence of VEGF (50 ng/ml), for 6 h. Alternatively, RMVEC were exposed to bevacizumab, L, Z or 5:1 lut:zeax, with VEGF (50 ng/ml), in the presence and absence of

GLX7013114 (1 μM). Images of tube formation were captured at × 10 magnification using a Zoe™ Cell Imager (BioRad). The number of joints and mesh size were calculated by using the Angiogenesis Analyser software in Image J as previously described (Lizunkova et al. 2019).

The experimental number is presented in the legend for each experiment and an average from two wells was assessed to represent an n of 1. Variance was assessed by using Bartlett's test. For data sets not reaching significance (Figures 1A-C, 2A,C,E, F) the Kruskal-Wallis test was used followed by Dunn's test. For all other data sets, differences among the means were tested for significance in all experiments by ANOVA with Tukey's range significance difference test. Significance was reached when $p < 0.05$. Values are presented as mean ± standard error mean (S.E.M.).

To understand the role of L, Z or the combined treatment of L:Z at a 5:1 ratio, compared to bevacizumab as a current therapy, we first sought to assess the cytotoxic effect of each treatment at a range of concentrations from 0.05 to 1 μg/μl. Whilst there was a trend for deceased viability at the highest concentration of L, Z, or 5:1 lut:zeax treatment (1 μg/μl), this did not reach significance (Figure 1A). In contrast, bevacizumab, the anti-VEGF antibody, resulted in a significant decrease in cell viability at 0.5 and 1 μg/μl to 62.2 ± 3.9% and 51.9 ± 3.3% (Figure 1A). Further studies for L, Z, 5:1 lut:zeax treatment and bevacizumab were performed at a non-cytotoxic but effective concentration of 0.25 μg/μl where there was no significant difference in viability between any of the treatment groups (Avery et al. 2006, Sonmez et al. 2008, Wang et al. 2014). We next studied the effect of these compounds on the ability of RMVEC to undergo VEGF-induced angiogenic processes. In the absence of VEGF, bevacizumab, L, Z and 5:1 lut:zeax treatment had no impact on cell proliferation (Figure 1B), adhesion (Figure 1C), migration (Figure 1D) and neovascularisation as measured by angiogenic potential (number of joints) and mesh size (Figure 1E-G). As anticipated, VEGF exposure significantly increased all of these measurements (Figure 1B-F) (Lizunkova et al. 2019). Interestingly, treatment with L or Z significantly reduced all of the VEGF-induced angiogenic processes studied, however these protective effects did not return values to those seen in vehicle-treated RMVEC (Figure 1B-G). In contrast, VEGF-induced increases in cell proliferation, adhesion, migration and mesh size were effectively blocked by exposure to either bevacizumab or 5:1 lut:zeax treatment (Figure 1B, C, D, G). Taken together, these findings show that independent treatment of L or Z can reduce VEGF-induced angiogenic processes in cells from the human retinal microvasculature. More importantly, these findings show that combination 5:1 lut:zeax treatment is highly effective in attenuating vascularisation in the retina, similar to a primary treatment for DR, bevacizumab.

One of the key clinical features of DR is oxidative stress associated with the increased inflammation and retinal injury (Calderon et al. 2017, He, M. et al. 2013, Tan, S. M. et al. 2013). Therefore to further understand the mechanism through which L and Z, and the combination treatment, protect against VEGF-induced angiogenic processes in human retinal microvascular endothelial cells, the next studies performed were to assess oxidative stress. As anticipated, exposure to VEGF significantly increased ROS accumulation in RMVEC over the 2 h period (Figure 2A). Interestingly, treatment with L, Z, 5:1 lut:zeax treatment or bevacizumab had no impact on cellular ROS levels (Figure 2A). Following exposure to both VEGF and the therapeutic treatments, L and Z significantly reduced, but did not block, VEGF-induced ROS accumulation in RMVEC (Figure 2B). In contrast, bevacizumab and 5:1 lut:zeax treatment effectively attenuated VEGF-induced cellular ROS to return levels to those seen in vehicle-treated RMVEC (Figure 2B). Levels of the endogenous antioxidant enzyme, glutathione (GSH), were measured as the primary protein responsible for clearing excess ROS and preventing oxidative stress. The observed VEGF-induced decrease in GSH expression was

significantly attenuated by exposure to bevacizumab and 5:1 lut:zeax treatment (Figure 2C). Whilst independent treatment with L also exerted a protective effect, to a lesser degree than combined treatment, Z exposure had no impact on cellular GSH expression (Figure 2C). These data demonstrate a clear role for 5:1 lut:zeax treatment in reducing VEGF-mediated oxidative stress in human retinal microvascular endothelial cells.

Nox4 is a major NADPH oxidase enzyme found in the retinal microvasculature and the only constitutively active Nox isoform (Serrander et al. 2007). In addition, Nox4 has been demonstrated to play a significant role in the development of retinal injury seen in DR (Appukuttan et al. 2018, Li et al. 2010, Wang et al. 2014). Therefore we next studied the effect of the potential therapeutic carotenoids on Nox4 activity. Exposure to bevacizumab, L, Z or 5:1 lut:zeax had no effect on Nox4 activity whereas exposure to VEGF alone significantly increased Nox4 activity (Figure 2D). Interestingly, all 4 therapeutic compounds significantly reduced VEGF-induced Nox4 activity but were unable to completely attenuate the effect (Figure 2D). To understand whether the reduction in VEGF-mediated Nox4 activity was associated with the protective effect of L, Z or 5:1 lut:zeax, the final experiments utilised the specific Nox4 inhibitor, GLX7013114. RMVEC were exposed to VEGF in the presence of either bevacizumab, L, Z or 5:1 lut:zeax treatment, and with or without GLX7013114. Cell proliferation and mesh size in Matrigel™ were assessed as markers for neovascularisation. Whilst the Nox4 inhibitor had no effect on cell proliferation or mesh size at baseline, following VEGF exposure, GLX7013114 significantly reduced, but not blocked, VEGF-induced angiogenic processes (Figure 2E and F). The protective effect of bevacizumab, in protecting against VEGF-induced proliferation and mesh formation, was not affected by Nox4 inhibition indicative of a Nox4-independent signal (Figure 2E and F). Interestingly, Nox4 inhibition significantly blocked the protective effect of L, Z and 5:1 lut:zeax treatment and prevented each from reversing VEGF-induced neovascularisation (Figure 2E and F). Taken together, these findings demonstrate that L, Z and 5:1 lut:zeax therapy may act as antioxidants, in VEGF-treated cells, to reduce angiogenic processes in a Nox4-dependent manner.

In the present study, we demonstrate the effect of the carotenoids L and Z, independently and in combination, on neovascularisation processes in human retinal microvascular endothelial cells. Our findings show that, in a VEGF-induced *in vitro* model of the eye linked to DR, both L and Z attenuate the neovascularisation processes of proliferation, adhesion, migration and tube formation similar to a current retinopathy therapy, bevacizumab. Interestingly, a combination therapy of L and Z (5:1 ratio) which mimics commercially-available supplements, such as Macushield (Akuffo et al. 2015, Crosby-Nwaobi et al. 2016), is more effective in blunting VEGF-mediated neovascularisation than independent treatment with each carotenoid. Furthermore, L and Z treatment, independently or in combination, blocked VEGF-mediated oxidative stress and Nox4 activity. Finally, we demonstrate that Nox4 activation is able to reverse the protective effect of the carotenoids on endothelial cell proliferation and tube formation. Taken together, these data show that L and Z can exert Nox4-dependent antioxidant effects which attenuate VEGF-induced neovascularisation. This study provides evidence that L and Z attenuate neovascularisation in an *in vitro* model of retinal injury linked to retinopathy. Findings demonstrate the role that Nox4 plays in regulating this protective effect of carotenoids and identifies a novel molecular target in the treatment of patients with retinopathy.

Patients who display retinal neovascularisation, for example, those with diabetic macular oedema in DR will be treated using intravital injection of anti-VEGF treatments (Ferrara et al. 2004, Heier et al.

2006). In the present study, we utilised one such treatment, a full-length anti-VEGF monoclonal antibody called bevacizumab, as a therapeutic comparison to lutein and zeaxanthin supplementation. Bevacizumab has been demonstrated to be effective in causing endothelial cell apoptosis which results in a loss of vascular endothelium within neovascular membranes and regression of abnormal neovascularisation in the retina of diabetic patients (Ababneh et al. 2013, Han et al. 2012). *In vitro* studies demonstrate that bevacizumab decreases VEGF-induced tube formation in retinal endothelial cells (Palanisamy et al. 2019). Our studies demonstrate that exposure of human retinal microvascular endothelial cells to varying concentrations of bevacizumab, in the absence of VEGF, causes cell death at concentrations of 0.5 and 1 µg/µl. In contrast, L, Z, or combination treatment had no impact on cell viability at baseline conditions indicating that these carotenoids may offer a less toxic approach than current anti-VEGF therapy. At present, a small percentage of patients receiving conventional anti-VEGF therapy can suffer from complications associated with neurodegeneration of healthy vessels and retinal detachment (Coney. 2019). In work presented here, we show that L, Z or 5:1 lut:zeax treatment have no impact on neovascularisation processes – proliferation, adhesion, migration and tube formation – in the absence of VEGF. These studies indicate that L and Z are working through a mechanism which blocks VEGF, and that healthy vasculature is not be affected by exposure to these carotenoids, either independently or in combination. Therefore L and Z may represent a therapeutic approach which could be preferable to other current anti-VEGF therapies. Whilst the human cell line model of retinopathy is a great tool to establish molecular mechanisms related to DR, further studies are essential to establish the role of L and Z in a physiological setting using *in vivo* techniques. Such studies include the use of microscopy with retinal whole mounts stained for endothelial cells using the WBN/Kob genetic or hypoxia inducible mouse models which display capillary occlusion, retinal ischaemia and neovascularisation characteristics of the disease (Olivares et al. 2017, Sun, Q. et al. 2020). Such studies would demonstrate the protective effect of L and Z combination treatment *in vivo* and link closely to the clinical studies with these micronutrients.

There have been several clinical studies which implicate a variety of dietary components in improving visual acuity in settings of AMD. These studies, such as the Blue Mountain Study, CARMIS and AREDS2, show that dietary supplementation with L and Z, amongst other micronutrients, reduce the risk of developing neovascular AMD (Chew et al. 2013, Dawczynski et al. 2013, Piermarocchi et al. 2012, Tan, J. S. et al. 2008). Despite these findings, there are limited studies which assess this efficacy or the protective mechanisms at a vascular level in these patients (Hu et al. 2011). Our findings demonstrate a protective, but not ablating, effect of either L or Z in settings of VEGF-mediated neovascularisation. Interestingly, when administered as a combination therapy, at 5:1 lut:zeax ratio, complete attenuation of VEGF-induced neovascular processes was observed. This indicates a therapeutic effect of this ratio of L and Z which is enhanced when administered in combination which is not surprising given that commercially-available supplements such as Macushield, are comprised of 5:1 ratio of L to Z (Akuffo et al. 2015, Crosby-Nwaobi et al. 2016). Bioavailability data indicates that there is a significant increase in plasma L (from 0.372 to 3.163 µg/dL) and Z (0.117 to 0.391 µg/dL) concentrations, following 12 week administration of 5:1 ratio of supplement (Juturu, et al. 2015). Of this large increase in carotenoid concentration, ocular tissues including the retina contain around 100-fold higher levels of L and Z compared to other tissues (Handelman et al. 1988, Landrum et al. 1997). These values correspond to around 0.25 µg/µl L and 0.048 µg/µl Z in the retina following 5:1 lut:zeax supplementation et al. 2015). In the present study, we used a concentration of 0.25 µg/µl for L, Z and the combination therapy, based on cytotoxicity study using a current anti-VEGF therapy, bevacizumab, which was used as a therapeutic comparison.

This converts to a concentration of 0.042 µg/µl Z and 0.208 µg/µl L which is physiologically-relevant for the retina following supplementation. Findings from this study can therefore be linked to supplementation studies which confer that L:Z, administered in combination at a 5:1 ratio, are protective against VEGF-induced neovascularisation processes.

The pathology of neovascularisation in the eye is generally linked with hyperglycaemia-induced oxidative stress. As such, micronutrients and vitamins which exert an antioxidant effect, in reducing oxidative stress in the retina, represent a potential therapeutic approach. Whilst association studies link dietary vitamin C and E with reduced markers of DR, multi-oxidant clinical trials show no significant improvement of the disease (Tabatabaei-Malazy et al. 2019). In the present study, we demonstrate the antioxidant properties of L, Z and combination therapy, in retinal microvascular endothelial cells, in settings of VEGF-induced oxidative stress. This is the first study performed in the retinal endothelium to show this effect however findings are in keeping with *in vitro* studies in the retinal pigment epithelium. Here, L has been shown to activate nuclear translocation of Nrf2 to reduce high glucose- or hypoxia-induced oxidative stress (Gong et al. 2017, Shivarudrappa and Ponesakki. 2019). In the choroidal vasculature, L supplementation, alongside consumption of long-chain polyunsaturated fatty acids, is associated with reduced Nox4-mediated oxidative stress (Yanai et al. 2018). Interestingly, Nox4 is the predominant Nox enzyme found in the retina, and has been linked to the onset of DR (Appukuttan et al. 2018, Li et al. 2010, Wang et al. 2014). In the present study, we demonstrate that L, Z and combination treatment lower ROS accumulation and Nox4 activity. Using the Nox4 inhibitor (GLX7013114), we further show that carotenoid-mediated protection against neovascularisation, in settings of VEGF, is dependent on Nox4 activity. This is in contrast to bevacizumab, where the protective effect of the humanised antibody on VEGF-mediated neovascularisation is unaffected by Nox4 activation. These findings represent a key difference between L and Z therapeutic approach and the typical anti-VEGF therapies used routinely. Furthermore, these studies indicate Nox4 as a key therapeutic target to treat the excessive retinal vascularisation seen in patients with DR. It is worth noting, however, that there are other Nox isoforms present within the retina which may play a role in regulating neovascularisation, such as Nox1 and Nox2 (Serrander et al. 2007). Indeed, in prostate cancers, Nox1 has been shown to trigger the angiogenesis switch whilst Nox2-derived ROS has been identified to mediate VEGF-induced migration (Arbiser et al. 2002, Ushio-Fukai et al. 1996). Therefore, further studies are needed to establish whether other Nox isoforms are mediated by L and Z to attenuate VEGF-induced neovascularisation and impact retinopathy.

Taken together, the present studies utilise a well-established *in vitro* model of retinopathy, and mirror findings from clinical studies such as AREDS2. Further work is, however, needed to establish the mechanism through which L and Z regulate Nox4 activity and understand how this can be translated to a more robust therapeutic approach for patients with DR.

Figure legends

Figure 1: Lutein and zeaxanthin protect against VEGF-induced angiogenic processes in human retinal microvascular endothelial cells. Endothelial cells were exposed to Bevacizumab, Lutein, Zeaxanthin, or a combination of lutein and zeaxanthin (5:1 lut:zeax), in the presence (closed bars) and absence (open bars) of VEGF (50 ng/ml). Cell viability (A), proliferation (B), and adhesion (C) were measured by MTT assay and cell count. Cell migration (D) was assessed by scratch assay, and neovascularisation (E, F and G) was measured using Matrigel™ assay. Representative images of Matrigel™ assay were captured in the presence and absence of VEGF (E) and quantification was

performed (F and G). Images were captured at x20 magnification, scale bar 200 µm. n=5, *p<0.05 versus 0 µg/µl (A) or vehicle (H_2O) (B-F), $^{\#}$p<0.05 versus VEGF.

Figure 2: Lutein and zeaxanthin reduce VEGF-induced oxidative stress and Nox4 activity to attenuate angiogenic processes. *Panels A-D:* Endothelial cells were exposed to Bevacizumab, Lutein, Zeaxanthin, or a combination of lutein and zeaxanthin (5:1 lut:zeax), in the presence (closed bars) and absence (open bars) of VEGF (50 ng/ml). ROS production (A and B), GSH expression (C) and Nox4 activity (D) were assessed using DCFDA, monochlorobimane dye, and specific ELISA kit respectively. *Panels E and F:* Endothelial cells were exposed to Bevacizumab, VEGF, Lutein, Zeaxanthin, or a combination of lutein and zeaxanthin (5:1 lut:zeax), in the presence (closed bars) and absence (open bars) of the Nox4 inhibitor, GLX7013114 (1 µM) or the vehicle (DMSO). Cell proliferation (E) and neovascularisation (F) were measured using cell count and Matrigel™ assay respectively. n=5, *p<0.05 versus vehicle for VEGF (H_2O), $^{\#}$p<0.05 versus VEGF, or $^{\delta}$p<0.05 versus vehicle for GLX7013114 (DMSO). .

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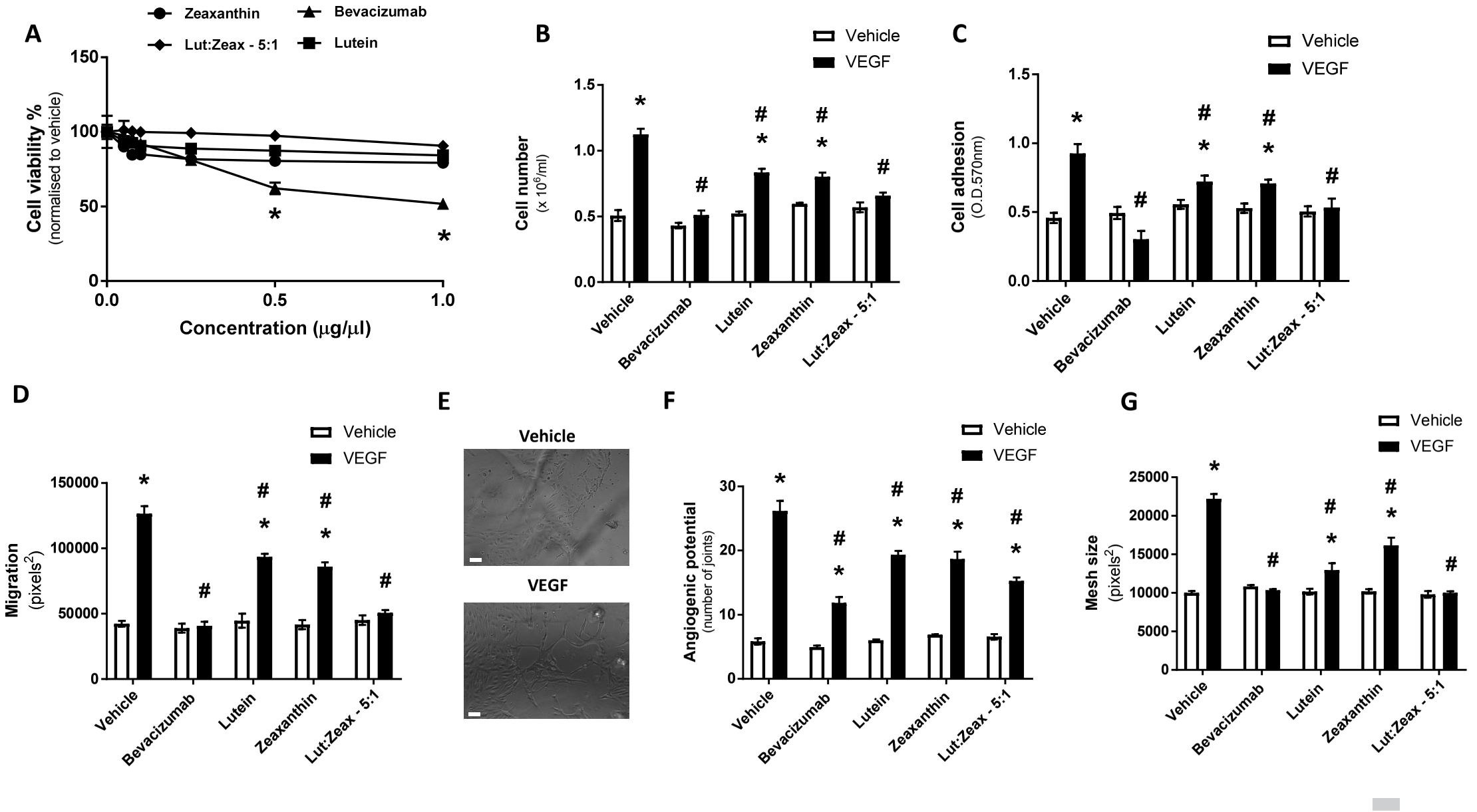
Figure 1


A
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Bevacizumab
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Lutein
Cell viability %
(normalised to vehicle)
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Zeaxanthin
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Cell adhesion
(O.D.570nm)
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Vehicle
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Angiogenic potential
(number of joints)
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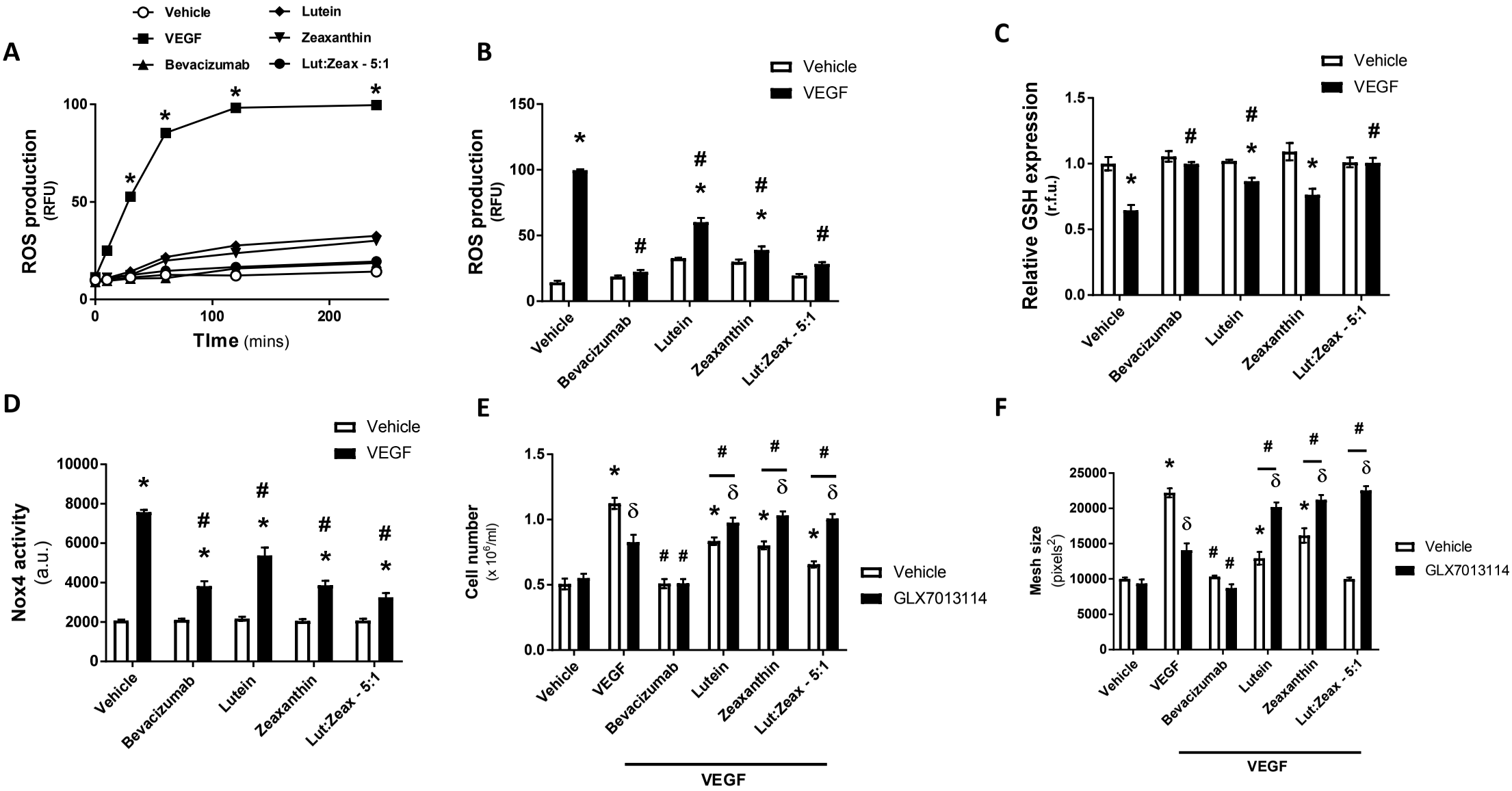
Figure 2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C
Vehicle
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Relative GSH expression (r.f.u.)
Vehicle
Bevacizumab
Lutein
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Lut:Zeax - 5:1
D
Vehicle
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Nox4 activity (a.u.)
Vehicle
Bevacizumab
Lutein
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Lut:Zeax - 5:1
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Cell number (x 10^6/ml)
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