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Corresponding Author: Dr. Miguel Angel Medina,

Corresponding Author's Institution: University of Málaga

First Author: Javier A García-Vilas

Order of Authors: Javier A García-Vilas; Ana R Quesada; Miguel Ángel Medina

Abstract: The health benefits of olive oil are attributed to their bioactive compounds, such as hydroxytyrosol. Previously, we demonstrated that hydroxytyrosol inhibits angiogenesis in vitro. The present study aimed to: i) get further insight into the effects of hydroxytyrosol on extracellular matrix remodeling; and ii) test whether hydroxytyrosol is able to inhibit angiogenesis ex vivo and in vivo. Hydroxytyrosol induced a shift toward inhibition of proteolysis in endothelial cells, with decreased expression of extracellular matrix remodeling-enzyme coding genes and increased levels of some of their inhibitors. Furthermore, this work demonstrated that hydroxytyrosol, at concentrations within the range of its content in virgin olive oil that can be absorbed from moderate and sustained virgin olive oil consumption, is a strong inhibitor of angiogenesis ex vivo and in vivo. These results suggest the need for translational studies to evaluate the potential use of hydroxytyrosol for angio-prevention and angiogenesis inhibition in clinical setting.

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Hydroxytyrosol targets extracellular matrix remodeling by endothelial cells and inhibits both *ex vivo* and *in vivo* angiogenesis

Javier A. García-Vilas[a,b], Ana R. Quesada[a,b] and Miguel Ángel Medina[a,b] *

[a]Universidad de Málaga, Andalucía Tech, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, and IBIMA (Biomedical Research Institute of Málaga)

[b]CIBER de Enfermedades Raras (CIBERER), E-29071 Málaga, Spain

***Corresponding author:** Dr. Miguel Ángel Medina, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, E-29071, Málaga. Phone: +34-952137132. Fax. +34-952131674. E-Mail: medina@uma.es

Running title: Antiangiogenic hydroxytyrosol

Abstract

The health benefits of olive oil are attributed to their bioactive compounds, such as hydroxytyrosol. Previously, we demonstrated that hydroxytyrosol inhibits angiogenesis *in vitro*. The present study aimed to: i) get further insight into the effects of hydroxytyrosol on extracellular matrix remodeling; and ii) test whether hydroxytyrosol is able to inhibit angiogenesis *ex vivo* and *in vivo*. Hydroxytyrosol induced a shift toward inhibition of proteolysis in endothelial cells, with decreased expression of extracellular matrix remodeling-enzyme coding genes and increased levels of some of their inhibitors. Furthermore, this work demonstrated that hydroxytyrosol, at concentrations within the range of its content in virgin olive oil that can be absorbed from moderate and sustained virgin olive oil consumption, is a strong inhibitor of angiogenesis *ex vivo* and *in vivo*. These results suggest the need for translational studies to evaluate the potential use of hydroxytyrosol for angio-prevention and angiogenesis inhibition in clinical setting.

Keywords: angiogenesis; aortic ring assay; bovine aorta endothelial cells (BAEC); chorioallantoic membrane (CAM) assay; hydroxytyrosol; matrix metalloproteinase (MMP); tissue inhibitor of metalloproteinase (TIMP); urokinase-type plasminogen activator (uPA)

1. Introduction

The phenolic compounds in extra virgin olive oil are bioactive compounds with well-documented beneficial effects (Fortes, García-Vilas, Quesada & Medina, 2012; Owen et al., 2000) these compounds are not essential in the sense that nutrients are. In fact, the European Union has authorized a health claim for polyphenols, based on consumption of olive oil polyphenols, for the protection of blood lipids from oxidative stress ("Commission Regulation (EU) 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. O.J. 2012, L136/1.," 2012). Hydroxytyrosol or 3,4-dihydroxyphenyl ethanol is claimed to be the most important health-related phenolic compound of virgin olive oil (Lopez de Las Hazas, Rubio, Kotronoulas, de la Torre, Sola & Motilva, 2015). Along with its cardioprotective effects (Mnafgui et al., 2015; Samuel, Thirunavukkarasu, Penumathsa, Paul & Maulik, 2008), beneficial effects of hydroxytyrosol for human health include its antifungal (Zoric et al., 2013), antidiabetic (Tutino, Orlando, Russo & Notarnicola, 2015; Zheng, et al., 2015), neuroprotective (De La Cruz, et al., 2015; Gallardo, Madrona, Palma-Valdes, Espartero & Santiago, 2015), anti-inflammatory (Fuccelli, Fabiani, Sepporta & Rosignoli, 2015; Persia, Mariani, Fogal & Penissi, 2014; Silva et al., 2015) and antitumoral (Granados-Principal et al., 2014; Sirianni et al., 2010; Sun, Luo, & Liu, 2014; Zhao et al., 2014) activities. In 2012, our group added the first description of hydroxytyrosol as an anti-angiogenic compound able to inhibit several key steps in the angiogenic process. In fact, we demonstrated that hydroxytyrosol (but not tyrosol) induced endothelial cell apoptosis and changes in cell cycle distribution as well as inhibiting endothelial cell proliferation, migration and differentiation into "capillary-like" tubes (Fortes et al., 2012). Furthermore, that work also identified matrix metalloproteinase 2 (MMP-2) as one of the molecular targets of the anti-angiogenic action caused by hydroxytyrosol. Since the publication of that article, additional data have been published regarding the roles of hydroxytyrosol as an anti-angiogenic compound. Several molecular targets have been identified contributing to the anti-angiogenic effects of hydroxytyrosol, including inhibition of MMP-9,

cyclooxygenase 2 and vascular endothelial growth factor receptor-2 (VEGFR-2) phosphorylation (Lamy, Ouanouki, Beliveau & Desrosiers, 2014; Scoditti et al., 2012; Scoditti et al., 2014). Furthermore, hydroxytyrosol has been recently shown to have protective effects against rheumatoid arthritis, an angiogenesis-dependent disease (Silva et al., 2015).

The identification of MMPs as molecular targets for hydroxytyrosol suggests that this compound can alter extracellular matrix remodeling (Fortes, García-Vilas, Quesada & Medina, 2012; Scoditti et al., 2014), a key step in the angiogenic process. Therefore, the first aim of the present work was to study the effects of hydroxytyrosol on key extracellular matrix remodeling enzymes expressed in endothelial cells. On the other hand, in spite of the recent efforts to get deep insights on the anti-angiogenic potential of hydroxytyrosol there is still no data available demonstrating its potential inhibitory effects in *ex vivo* or *in vivo* models of angiogenesis. The second aim of this work was to test the anti-angiogenic potential of hydroxytyrosol in the *ex vivo* aortic ring and the *in vivo* CAM angiogenesis models.

2. Materials and methods

2.1. Chemicals

Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis MO, USA). Cell culture media, penicillin, streptomycin and amphotericin were purchased from Biowhittaker (Walkersivlle, MD, USA). Fetal bovine serum (FBS) and human serum (HS) were products of Harlan-Seralb (Belton, United Kingdom). Plastics for cell culture were supplied by NUNC (Rockilde, Denmark) and VWR (West Chester, Pennsylvania, USA). Hydroxytyrosol was supplied by Extrasynthèse (Lyon, France).

2.2. Cell culture

Most of the procedures described in Materials and methods have been previously used by our research group for other studies (see, for instance Garcia-Vilas et al. 2013, Martínez-Poveda

et al. 2013). Bovine aorta endothelial cells (BAEC) were isolated from bovine aortic arches, as previously described (Gospodarowicz & Moran, 1975), and maintained in Dulbecco´s modified Eagle´s medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/ L), amphotericin (1.25 mg/L), 10% fetal bovine serum.

2.3. RNA isolation and purification and cDNA synthesis

Cells at 80% of confluence in 6-well plates were treated with or without 1mM of hydroxytyrosol for 24 h. After incubation, cells were harvested and washed (PBS). Total RNA was isolated with the GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the purchaser's instructions.

cDNA synthesis was carried out with the iScript cDNA synthesis kit (BioRad).

2.4. qPCR

For quantitative RT-PCR (qPCR), total RNA isolation and complementary DNA synthesis were performed as described above and PCR reactions were done using KAPA SYBR Fast Master Mix (2x) Universal (KAPA Biosystems) in an Eco Real-Time PCR System. qPCR was performed in triplicate for each sample in keeping with the manufacturer´s instructions. All qPCR data were normalized to GAPDH expression (Martínez-Poveda et al. 2013). Primers, amplicon size, and qPCR conditions for each gene are shown in Table 1.

2.5. Animals and ethical statement

All experimental procedures with animals were conducted in accordance with the Spanish Legislation (Real Decreto 53/2013, BOE, 34/-11421, 2013) in compliance with the European Community Directive 2010/63/EU regulating the use and care of laboratory animals. The

protocols were approved by the Ethics Committee for Animal Experiments of the University of Málaga.

2.6. Ex vivo *rat aortic ring assay*

Thoracic aortas were removed from 12 week old rats and immediately transferred to a culture dish containing DMEM. The perioaortic fibroadipodise tissue was carefully removed with fine microsdissecting forceps and iridectomy scissors paying special attention not to damage the aortic wall. Afterwards, 1-mm aortic rings were sectioned and embedded in a rat tail interstitial collagen gel (1.5 mg/mL) prepared by mixing 7.5 volumes of 2 mg/mL collagen, 1 volume of 10x HBBS, 1.5 volumes of 186 mM $NaHCO_3$ and 0.1 volumes of 1 M NaOH to adjust the pH to 7.4. Collagen gels containing the aortic rings were polymerized in cylindrical agarose wells and kept in triplicate at 37˚C in 60 mm diameter Petri dishes (bacteriological polysterene, Falcon, Becton Dickinson, Lincoln Park, New Yersey). Each Petri dish contained 6 mL of MCDB131 medium supplemented with 1% L-glutamine, 25 mM $NaHCO_3$, 100 U/mL penicillin, 100 µg/mL streptomycin, in presence of hydroxytyrosol or etanol (vehicle). Cultures were kept at 37˚C, 5% CO_2 in a humidified environment, and photographs were taken after 6, 9 and 14 days. The antiangiogenic response was quantified by microvessel counting according to published criteria (Nicosia & Ottinetti, 1990).

2.7. In vivo *chorioallantoic membrane (CAM) assay*

Fertilized chick eggs were incubated horizontally at 38°C in a humidified incubator, windowed by day 3 of incubation and processed by day 8. The indicated amount of hydroxytyrosol was added to a 1% solution of methylcellulose in water, and 10 µL drops of this solution were allowed to dry on a Teflon-coated surface in a laminar flow hood. Then, the methylcellulose disks were implanted on the CAMs, and the eggs were sealed with adhesive tape and returned to the incubator for 48 h (Garcia-Vilas et al. 2013). Negative controls were

always made with ethanol (vehicle) mixed with methylcellulose. After the reincubation, CAMs were examined under a stereomicroscope. The assay was scored as positive when two independent observers reported a significant reduction of vessels in the treated area.

2.8. Statistical analysis

For all of the assays, at least three independent experiments were carried out. Results are expressed as means ± SD. Statistical significance was determined by using Student´s paired simple test. Values of $p<0.05$ were considered to be significant.

3. Results

3.1. Hydroxytyrosol induces changes in the expression levels of genes involved in ECM remodeling in endothelial cells

To fulfil the first goal of our work, we analyzed by qPCR the effects of 1 mM hydroxytyrosol treatment of BAEC for 24 h on the expression levels of messenger corresponding to a number of genes involved in ECM remodeling. The primers, annealing temperature and amplicon sizes are summarized in Table 1. Table 2 summarizes the qPCR quantitative data for the 9 tested potential targets. Both MMP-1 and MMP-2 mRNA expression levels were drastically diminished by hydroxytyrosol, whereas three out of four tissue inhibitors of metalloproteinases (namely, TIMP-1, -2 and -4) mRNA expression levels were increased several fold. On the other hand, the mRNA levels for urokinase-type plasminogen activator (uPA), another ECM remodeling enzyme, were also drastically diminished upon hydroxytyrosol treatment, whereas the levels of the messenger for its specific receptor (uPAR) increased more than 20-fold. Finally, we could not detect any signal for plasminogen activator inhibitor 1 (PAI-1) mRNA.

3.2. Hydroxytyrosol has a very potent inhibitory effect on the ex vivo *rat aortic ring angiogenesis assay*

The second goal of this work was to test the anti-angiogenic potential of hydroxytyrosol in the *ex vivo* aortic ring and the *in vivo* CAM angiogenesis models. Figure 1 clearly shows that hydroxytyrosol was able to induce very strong inhibitory effects in the *ex vivo* aortic ring assay, even at concentrations much lower than those used previously by us to show its *in vitro* anti-angiogenic effects (Fortes, García-Vilas, Quesada & Medina, 2012). At 0.25 mM hydroxytyrosol the inhibition of microvessel outgrowth from the aortic rings was complete after 6, 9 and 14 days of incubation. At 0.125 mM hydroxytyrosol we observed outgrowing of proliferative endothelial cells from the aortic ring but these cells were not forming microvessel tubes. Furthermore, there were partial inhibitory effects for 62.5 µM hydroxytyrosol.

3.3. Hydroxytyrosol inhibits in vivo *angiogenesis*

Figure 2 shows that in untreated, control CAMs, blood vessels form a spatially-oriented and dense network of vascular structures with progressively smaller diameters as they branch. Hydroxytyrosol-treated CAMs showed inhibited angiogenesis, as revealed by an inhibition of new vessel formation within the area covered by the methylcellulose discs, a centrifugal growth of peripheral vessels, avoiding the treated area, and an overall decrease in vascular density. Table 3 summarizes the results obtained with the CAM assay. Positive, inhibitory effects were observed in 90% of eggs treated with 800 nmol of hydroxytyrosol. The inhibition was still observed in more than half of the CAMs treated with 400 nmol of hydroxytyrosol. For treatments with 200 and 100 nmol of hydroxytyrosol, 42% and 13% of the eggs scored positive. No inhibitory effect was observed in eggs treated with 50 nmol of hydroxytyrosol.

4. Discussion

As mentioned in the Introduction, a number of very different biological effects have been shown for hydroxytyrosol, underscoring its preventive and pharmacological potential. Our group demonstrated for the first time that hydroxytyrosol also behaves as an anti-angiogenic compound *in vitro* (Fortes, García-Vilas, Quesada & Medina, 2012). One of the molecular targets for this effect of hydroytyrosol was the extracellular remodeling enzyme MMP-2, which plays a key role in the basal membrane destruction needed for the migration and invasion of proliferative endothelial cells during the angiogenic process (Stetler-Stevenson, 1999). This result was consistent with the description by another independent research group of the suppression of MMP-9 expression by hydroxytyrosol in both human endothelial cells (Scoditti, et al., 2012) and activated human monocytes (Scoditti, et al., 2014). Our qPCR results (Table 2) indicate that, indeed, hydroxytyrosol seems to have a global stimulating effect on ECM remodeling by both decreasing the expression levels of genes coding for extracellular matrix remodeling enzymes (MMP-1, MMP-2, uPA) and increasing the levels of some of their inhibitors (TIMP-1, -2, and -4).

A particularly important molecular target of hydroxytyrosol is VEGFR-2, since it plays a key role as a master regulator of the pro-angiogenic phenotype. In fact, it has been shown that hydroxytyrosol is a very potent inhibitor of the specific autophosphorylation sites (Tyr951, Tyr1059, Tyr1175, and Tyr1214) of VEGFR-2, thus inhibiting angiogenesis (Lamy, Ouanouki, Beliveau, & Desrosiers, 2014). These and other molecular data strongly suggested that hydroxytyrosol might have protective effects on angiogenesis-dependent diseases. This seems to be the case for age-related macular degeneration and rheumatoid arthritis (Granner, Maloney, Antecka, Correa & Burnier, 2013; Silva et al., 2015). Nonetheless, there is still need additional pre-clinical data showing modulatory effects of hydroxytyrosol beyond the *in vitro* situation to boost the interest to test the hydroxytyrosol potential for its clinical use. In this context, both *ex vivo* and *in vivo* assays seem required and useful. To our knowledge, the only available study showing an additional and indirect evidence of the anti-angiogenic effect of hydroxytyrosol *in vivo* was the article showing that hydroxytyrosol suppresses the growth of human hepatocellular

carcinoma through the inactivation of both AKT and NF-kB pathways (Zhao et al., 2014). In that article, figure 5C shows a histogram with the quantification of microvessel density in orthotopic hepatocellular carcinoma tumors stained for the microvessel marker CD31. In the present work, we have used two very popular pre-clinical angiogenesis assays, namely, the *ex vivo* aortic ring assay and the *in vivo* CAM assay. The *ex* vivo aortic ring assay allows for the analysis of cell proliferation, migration, tubule-like formation, microvessel branching and perivascular recruitment and remodeling (Baker et al., 2012). The CAM assay is the most frequently used in vivo assay of angiogenesis (Ribatti, 2008). The results obtained to fulfil the second aim of the present work have contributed to provide clear and remarkable evidence that, indeed, the anti-angiogenic effects of hydroxytyrosol observed previously *in vitro* are strongly confirmed by both *ex vivo* and *in vivo* angiogenesis assays carried out in the present work (Figures 1 and 2, and Table 3).

Interestingly, the effects described in the present work were obtained with hydroxytyr0sol doses within the range described previously as absorbed from a sustained and moderate dose of virgin olive oil, similar to that corresponding to its daily intake in a typical Mediterranean diet (Miró-Casas et al., 2003).

The results presented in this work can be considered a step further toward translational studies to be carried out in the near future regarding the potential use of hydroxytyrosol in angioprevention and for the pharmacological inhibition of angiogenesis.

Conflict of interest

The authors declare that they have no financial or other conflict of interest.

Acknowledgements

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361 **Table 1**. Primers used for qPCR with indication of their respective annealing temperatures
362 and amplicon sizes.
363

Gene	Primers	**A**nnealing temperature (°C)	Amplicon size (bp)
MMP-1	Forward: gacttagtccagaaatacctg Reverse: caaagaattcctgcatttgc	60	121
MMP-2	Forward: gacatacatctttgctggagac Reverse: acgctcttcagactttggttct	60	200
TIMP-1	Forward: gggacaccagaagtcaacca Reverse: ggcttggaacccttatacatc	63	81
TIMP-2	Forward: aagcggtcagtgagaaggaa Reverse: tctcaggccctttgaacatc	65	112
TIMP-3	Forward: gcagcggaccacaacagcta Reverse: ccggatcacgatgtcggagt	68	150
TIMP-4	Forward: agggagagcctgaatcatca Reverse: gcactgcatagcaagtggtg	65	68
uPA	Forward: cgccacacactgcttcatg Reverse: ccccttgcgtgttggagtt	60	310
uPAR	Forward: gcccaatcctggagcttga Reverse: tccccttgcagctgtaacact	60	63
PAI-1	Forward: gcacaaccccacaggaaca Reverse: gtcccgatgaaggcgtcttt	65	81

Table 2. Relative expression values of mRNAs for some extracellular matrix remodelling enzymes and their inhibitors in BAEC treated with hydroxytyrosol.

	MMP-1	MMP-2	TIMP-1	TIMP-2	TIMP-3	TIMP-4	uPA	uPAR	PAI-1
BAEC	16.9 ± 0.5	0.28 ± 0.2	3857.3 ±537.1	418.0 ±123.9	14.76 ± 1.47	377.6 ± 209.6	20.95 ± 12.1	2195.8 ± 433.0	-

qPCR was carried out as described in Materials and Methods. All qPCR data were normalized with GAPDH expression levels. Data are given as means±S.D. and they are percentages of expression taking the corresponding expression values in control, untreated cells as 100%.

373 **Table 3.** Inhibition of *in vivo* angiogenesis by hydroxytyrosol (HT) as determined by the

374 CAM assay.

HT (nmol/CAM)	Positive/Total	Inhibition (%)
0	0/8	0
50	0/6	0
100	1/8	13
200	5/12	42
400	5/8	63
600	5/7	71
800	9/10	90

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Figure legends

Fig. 1. Hydroxytyrosol inhibits microvessel outgrowth in the *ex vivo* rat aortic ring assay. (**A**) Representative samples of the aortic ring without or with treatment. (**B**) Quantification of the area occupied by new microvessels in controls, controls treated with VEGF and rings treated with 31.2 µM or 62.5 µM hydroxytyrosol and VEGF. The results are the mean ± SD of three different assays.

Fig. 2. Hydroxytyrol inhibits angiogenesis *in vivo* in the CAM assay. Arrows point rebound of vessels outward from the treated area. Asterisks indicate disrupted vessels.

Figure 1

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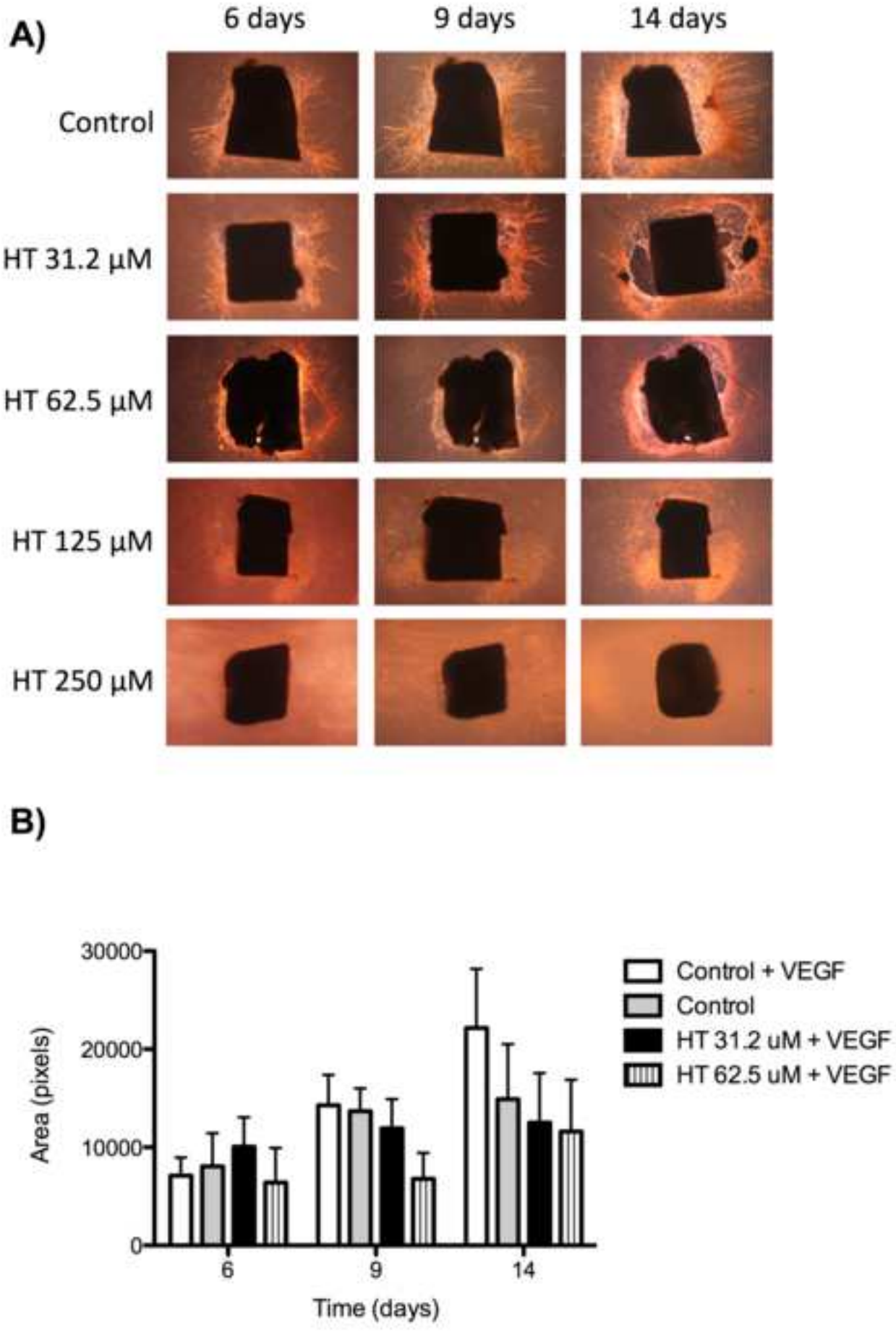

Figure 2

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