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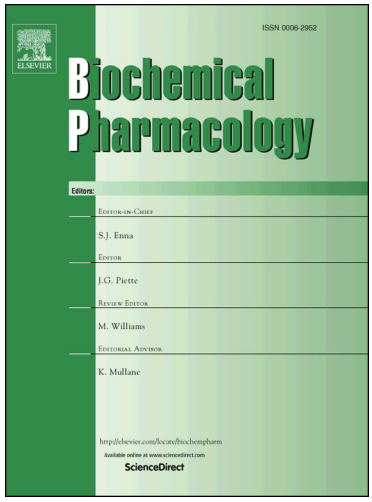

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The effects of flavonoids on human first trimester trophoblast spheroidal stem cell self-renewal, invasion and JNK/p38 MAPK activation: Understanding the cytoprotective effects of these phytonutrients against oxidative stress

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Abstract

Adequate invasion and complete remodelling of the maternal spiral arteries by the invading extravillous trophoblasts are the major determinants of a successful pregnancy. Increase in oxidative stress during pregnancy has been linked to the reduction in trophoblast invasion and incomplete conversion of the maternal spiral arteries, resulting in pregnancy complications such as pre-eclampsia, intrauterine growth restriction, and spontaneous miscarriages resulting in foetal/maternal mortality. The use of antioxidant therapy (vitamin C and E) and other preventative treatments (such as low dose aspirin) have been ineffective in preventing pre-eclampsia. Also, as the majority of antihypertensive drugs pose side effects, choosing an appropriate treatment would depend upon the efficacy and safety of mother/foetus. Since pre-eclampsia is mainly linked to placental oxidative stress, new diet-based antioxidants can be of use to prevent this condition. The antioxidant properties of flavonoids (naturally occurring phenolic compounds which are ubiquitously distributed in fruits and vegetables) have been well documented in non-trophoblast cells. Therefore, this study aimed to investigate the effects of flavonoids (quercetin, hesperidin) and their metabolites (Quercetin 3-O-β-glucuronide and hesperetin), either alone or in combination, on first trimester trophoblast cell line HTR-8/SVneo during oxidative stress. The data obtained from this study indicate that selected flavonoids, their respective metabolites significantly reduced the levels of reduced glutathione ($p<0.0001$) during HR-induced oxidative stress. These flavonoids also inhibited the activation of pro-apoptotic kinases (p38 MAPK and c-Jun N-terminal kinase) during HR-induced phosphorylation. In addition, they enhanced spheroid stem-like cell generation from HTR8/SVneo cells, aiding their invasion. Our data suggest that dietary intake of food rich in quercetin or hesperidin during early pregnancy can significantly improve trophoblast (placenta) health and function against oxidative stress.

Keywords

Glutathione; Antioxidant; Reactive oxygen species; Extravillous trophoblast cells; Placenta; quercetin, hesperidin, hesperetin, Q3G, HTR-8/SVneo cells, flavonoids, pre-eclampsia, spheroid, stem cell, invasion

Abbreviations

IUGR, intrauterine growth restriction; ROS, reactive oxygen species; GSSG, oxidised glutathione; GSH, reduced glutathione; MAPK, mitogen-activation protein kinase; SAPK, stress-activated protein kinases; JNK, c-Jun N-terminal kinase; PE, pre-eclampsia; 3D, three dimensions, Q3G; Quercetin 3-O-β-glucuronide.

1. Introduction

The placenta is a highly specialised multifunctional organ that is responsible for maintaining the development and growth of the foetus, by acting as a medium of exchange between the mother and developing foetus [1–3]. Therefore, the proper development and function of the placenta is crucial to the health of the mother and developing foetus [1]. During early gestation, normal placental development and function is dependent on the coordinated differentiation of the cytotrophoblast (which gives rise to the extravillous trophoblast) [4,5]. First trimester trophoblast cells, are known to maintain their highly invasive and proliferative phenotype [5]. Amongst these, first trimester extravillous trophoblast (EVT) cells are the highly invasive, proliferative and migratory subtype of the cytotrophoblast. They are mainly responsible for the physiological remodelling of the maternal spiral arteries into a high flow, low resistance system [6,7] which is essential for placental development/function and to support the well-being of the mother and foetus during gestation. However, defects in these processes, such as the reduced/shallow invasion of the extravillous trophoblast cells, will result in insufficient spiral artery remodelling. These defects (i.e. insufficient invasion and incomplete maternal spiral artery remodelling) have been linked to placental oxidative stress that leads to several maternal complications [8].

Oxidative stress can be defined as the imbalance in the generation and accumulation of reactive oxygen species (ROS) in cells/tissues, and the ability of antioxidants to detoxify them. It mainly results from increased production of ROS and/or decreased capacity of the antioxidant defence system to tackle normal ROS production [9,10]. Considerable evidence has linked oxidative stress to the pathophysiology of several chronic illnesses including cardiovascular disorders, neurological complications, cancers, respiratory disease, rheumatoid arthritis as well pregnancy-related disorders [10,11].

Interestingly, pregnancy can be considered as a state of oxidative stress due to the increased placental mitochondrial activity and production of ROS as low/moderate levels of oxidative stress is essential for the normal development of foetal growth [12,13]. Increased placental oxidative stress has been linked to the pathophysiology of pregnancy complications such as foetal growth restriction, pre-eclampsia (PE), maternal/foetal immune disturbance, and miscarriage [1,14]. PE is a major disease of human pregnancy, characterised by hypertension (160/110 mmHg) and proteinuria (>300 mg/24 h) mainly developing after 20 weeks of gestation [8]. It is estimated to affect about 3 – 14 % of pregnant women worldwide and the incidence varies according to geographical location, nutrition, and race/ethnicity [8]. It is the leading cause of perinatal and maternal mortality and morbidity worldwide [15].

Although the exact cause of PE is still unknown, considerable evidence has shown that an increase in placental oxidative stress as a result of severe hypoxia, especially hypoxia reoxygenation (HR), plays a major role in the pathogenesis of PE [3,16]. To date, the only effective solution to PE is preterm delivery or termination of pregnancy. Therefore, prevention and treatment are of major clinical importance. Research to date has focused on reducing and/or preventing placental oxidative stress using antioxidant (non-enzymatic) therapy such as vitamin C and E, either alone or in combination [8,17]. It is notable that the craving of fruits and vegetable that are rich in flavonoids (naturally occurring phenolic compounds with significant antioxidant and chelating properties) has been reported during pregnancy [18–20]. Although, it is possible for researchers to study the effects of naturally occurring edible plant products and dietary supplementations as a preventive measure against PE [8,17], due to ethical constraints, it is impossible to study their direct effects on the developing placenta or early trimester trophoblast cells. Therefore, the cellular effects of these known chemicals from edible sources can only be studied *in vitro* using transformed trophoblast cell lines. Especially, multicellular spheroids have been proposed to be a suitable *in vivo* model for investigating the

effects of drugs [21]. Three dimensional (3D) spheroidal culture offers many physiological advantages for the testing of drug delivery and toxicity such as cell-cell interactions, cell-matrix contacts and 3D shape similar to that of tissue [21,22]. We previously reported that flavonoids (quercetin and hesperidin), their metabolites (Quercetin 3-O-β-glucuronide {Q3G} and hesperetin) alone or in combination significantly triggered protective effects in the human first trimester trophoblast cell line (HTR-8/SVneo) against HR-induced oxidative stress/apoptosis [19]. In the present study, we investigated the effects of quercetin, hesperidin, and their respective metabolites alone or in combination on HR-induced glutathione (GSH) levels, p38 mitogen-activation protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) activation. We also looked at their effects on invasion of normal HTR-8/SVneo cells and their ability to transform spheroidal stem cell formation/invasion. We found that pre-treatment with the indicated flavonoids and their metabolites, alone or in combination, prior to HR insult significantly increased glutathione levels, inhibited JNK and p38 MAPK activation, increased HTR-8/SVneo invasive capacity and enhanced spheroidal stem cell growth/invasion.

Understanding the relationship between placental development, increased oxidative stress and the cytoprotective effects of dietary flavonoids during early gestation may contribute to the advancement of new concepts in preventing pregnancy related complications.

We were particularly interested in quercetin and hesperidin, and their metabolites because these two chemicals are found in fruits and vegetables that are been craved by many pregnant women worldwide [17,18].

2. Materials and Methods

2.1. Cell Culture and hypoxia reoxygenation insult

Transformed human first trimester trophoblast cell line (HTR-8/SVneo) was kindly gifted by Dr Charles Graham, Queen's University, Canada (passages used ranged between 33-50). The

cells were grown and maintained in RPMI-1640 with L-glutamine (Lonza, UK) supplemented with 10% (v/v) foetal bovine serum (FBS; Gibco®, UK), penicillin (100 U/ml) (Lonza, UK) and streptomycin (100 µg/ml) (Lonza, UK) and cultured in a humidified incubator (5% CO_2 at 37 ºC) until 80–90% confluent. Cells were passaged and further sub-cultured as described previously [19].

Flavonoids/metabolites used in this study were purchased from (Sigma-Aldrich®, UK) and their purity was as follows: quercetin (≥95%), hesperidin (≥80%), hesperetin (≥98%) and quercetin 3-glucuronide (≥90%). All experiments were carried out using the same batch numbers. Stock solutions (10 mM) of flavonoids (quercetin and hesperidin) and their metabolites (Q3G and hesperetin) were freshly prepared in dimethyl sulfoxide (DMSO; Fisher Scientific, UK). Final flavonoid/metabolite concentrations ranged between 1 and 3 µM in RPMI-1640 growth media [with a final DMSO concentration of 0.1% (v/v)]. Vehicle control studies using DMSO did not reveal an effect of DMSO on MTT cell viability assays.

HR-induced oxidative stress was achieved as described previously [19], by exposing HTR-8/SVneo cells to 2 h hypoxia followed by 6 h reoxygenation in serum- and glucose free RPMI-1640 media (Gibco, UK) in a modular incubator chamber was purchased from Billups-Rothenberg Inc. (San Diego, USA). A gas mixture of 0.2% O_2, 5% CO_2 and 94.8% N_2 was bought from BOC Limited (Nottingham, UK) and certified by HiQ® (Nottingham, UK). HTR-8/SVneo cells were routinely checked for mycoplasma using PCR. The authenticity and the identity of this cell line was verified by the curator (Dr Charles Graham) and the European Collection of Authenticated Cell Cultures (ECACC, Porton Down, UK)

2.2.Measurement of GSH

GSH detection and quantification were carried out using a GSG/GSSG-Glo™ assay (Promega, UK). HTR-8/SVneo cells were cultured in a 96-well black with clear bottom tissue culture plate (Flacon®; Fisher Scientific, UK) at a density of 2 x 10^4 cells/well and incubated overnight before treating with quercetin or hesperidin (3 µM), their metabolites (Q3G or hesperetin) (1 µM) alone or in combination (3 + 1 µM) for 24 h before exposure to HR-induced oxidative stress. Afterwards, the media was aspirated and replaced with either total glutathione lysis reagent or oxidized glutathione lysis reagent, after which cells were agitated at room temperature for 5 min. The plate was then incubated at room temperature for 30 min before the addition of luciferin generation reagent. Luminescence was measure using a FLUIstar Omega plate reader (BMG LABTECH, UK) and GSH quantification was calculated according to manufacturer's guidelines.

2.3. Western blot analysis

To determine the effect of flavonoid pre-treatment on the activation/inhibition of SAPK/JNK and p38 MAPK, western blot analysis was performed using protein extracts prepared from HTR-8/SVneo cells following HR-induced oxidative stress. HTR-8/SVneo cells were cultured in T75 flasks to a confluency of 70-80% before treating with flavonoids prior to HR-induced oxidative stress. Total cell lystates were extracted in Pierce® RIPA buffer (ThermoScientific, UK) supplemented with Roche cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, UK) and phosphatase inhibitor cocktail (Roche PhosSTOP™; Sigma-Aldrich, UK) according to manufacturer's guidelines. Protein concentration was determined using the Bicinchoninic Acid (BCA; Sigma-Aldrich, UK) assay. Afterwards, equal amounts (30 µg protein) of samples were loaded onto 12.5% sodium dodecyl sulphate polyacrylamide electrophoresis gels (ProtoGel®, National Diagnostics, UK). The gels were then electro-blotted onto 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories Ltd, UK) by wet-transfer using Mini-PROTEAN® Tetra electrophoresis system (Bio-Rad Laboratories Ltd, UK). Following

transfer, the membranes were blocked for 1 h in tris-buffered saline (pH 7.5) containing 3% w/v bovine serum albumin (BSA) and 0.1% v/v Tween 20. After blocking, the membranes were incubated with the following primary antibodies (1:1000 dilutions unless otherwise indicated): rabbit monoclonal anti-JNK1/2/3; ab179461 (Abcam, UK), rabbit monoclonal phospho SAPK/JNK; 4668S (Cell Signalling Technology, UK), rabbit monoclonal p38 MAPK; 8690S and phospho p38 MAPK; 4511s (Cell Signalling Technology, UK) overnight at 4 ºC. After incubation, the membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase conjugated secondary antibody; ab6721 (Goat Anti-rabbit (1:2000; Abcam, UK). Protein bands were detected by EZ-chemiluminescence detection kit (Geneflow Ltd, UK) and the chemiluminescence signal was obtained using a FijuFilm LAS 4000 imager. Images of the bands were digitized, and densitometry performed using Li-Cor image studio™ lite (Li-Cor®, UK). To confirm equal loading of samples on the gel, the membranes were stripped and re-probed with either β-actin; ab8227 (1:3000; Abcam, UK) or β-tubulin; 2128s (1:1000; Cell Signalling Technology, UK) as a loading control.

2.4. *In Vitro* cell invasion assay

Corning® BioCoat™ (UK) tumour invasion system was used to assess the effects of flavonoids, their metabolites or combinations on HTR-8/SVneo cells prior to HR-induced oxidative stress following manufacturer's guidelines. Briefly, suspensions of HTR-8/SVneo cells (in serum-free RPMI-1640 media) obtained following HR-induced oxidative stress were labelled with 5 µM CellTrace™ CFSE dye (Molecular Probe®, UK) according to the manufacturer's instructions. Pre-labelled HTR-8/SVneo cell suspensions (1.25×10^4 cells/well) were placed in each well of the apical chamber of both the coated (invasion plate) and uncoated (migration plate) 96-multiwell plates (Corning, UK) Serum-free RPMI 1640 and growth media containing 5% (v/v) FBS were added to the basal chamber of both plates, serving as the chemoattractant. After incubation for 24 h in a humidified tissue culture incubator (37 ºC, 5% CO_2), the Matrigel

coated and uncoated membranes were detached, and the invaded/migrated cells were imaged using an EVOS FL microscope (ThermoFisher Scientific, UK). For quantification and analysis, cell counting was carried out by WimCounting (Wimasis Image Analysis, Germany) and percentage invasion was calculated by dividing the number of invaded cells by the number of migrated cells multiplied by 100.

2.5. Spheroid growth and invasion

The effects of HR-induced oxidative stress on the ability of HTR-8/SVneo cells to generate spheroidal stem cells and their invasive capabilities were investigated using soft-agar colony formation and Cultrex® 3D spheroid base membrane extract (BME) cell invasion system (UK)

For spheroid growth, a soft-agar colony formation assay was carried out by dispensing a mixture (1:1) of 1% (w/v) Difco™ Noble Agar (BD Biosciences, USA) and RPMI-160 growth media containing 20% (v/v) FBS into a 6-well plate to form the basal layer. The upper layer was made up with a mixture (1:1) of 0.7% (w/v) Agarose (Sigma-Aldrich, UK) and HTR-8/SVneo cell suspension (2.5×10^3 cells with or without flavonoid treatment prior to HR-induced oxidative stress). The plate was kept in the humidified tissue culture incubator and medium was replaced twice a week. Spheroid morphology and growth was observed using an inverted microscope (Nikon Eclipse, TS100, UK) attached with a Nikon DS-Fi2 camera. Spheroid growth area (µm) was measured by WimColony (Wimasis Image Analysis, Germany).

For spheroid invasion, HTR-8/SVneo cells were cultured in T75 flasks, treated and exposed to oxidative stress as described previously in section 2.4. The assay was carried out following the manufacturer's guidelines (Cultrex® 3D spheroid cell invasion assay Amsbio, UK). HTR-8/SVneo cell suspension (3×10^3 cells) after HR insult was prepared in 1X spheroid formation extracellular matrix cocktail (AMS Biotechnology, UK) before adding to a 3D culture qualified

96-well spheroid formation plate. After 72 h of incubation (37 ºC, 5% CO_2), spheroid invasion matrix was added to the 3D culture qualified 96-well spheroid formation plate and the first image was taken using a confocal microscope (LAS AF, Leica Microsystems, UK). ImageJ (NIH Image, uk) was used to measure the area of invasion after 72 h.

2.6. Statistical analysis

All experiments were carried out at least three separate and distinct experiments times, each in triplicate. Data are expressed as means ± SEM (standard error of mean) calculated using GraphPad® Prism-7 Software. For each variable tested, one-way analysis of variance (ANOVA) was performed followed by Dunnett's/Turkey's multiple comparisons test to determine any differences between the means. A significant effect was indicated by $P < 0.05$.

3. Results

3.1. Effects of flavonoids/metabolites on cellular GSH levels.

Data from our previous study showed that HR significantly elevated the oxidised glutathione (GSSG) levels whilst the introduction of either quercetin, hesperetin or their metabolites significantly reduced GSSG levels [19]. Therefore, it was crucial to assess the GSH level in order to understand the effects of HR in the presence or absence of flavonoids on GSH:GSSG ratio (a major indicator of oxidative stress). HR-induced oxidative stress significantly lowered the levels of GSH in HTR-8/SVneo cells (Fig. 1). Our results demonstrate that 24 h pre-treatment with either 3 µM quercetin or 3 µM hesperidin were associated with significant increase in the levels of GSH when compared to untreated (normoxic) cells (Fig. 1A and 1D). Similarly, their metabolites, Q3G (1 µM) and hesperetin (1 µM), were also associated with significant elevation in GSH levels when compared to both HR untreated and control cells as shown in Fig. 1B and 1E respectively. In addition, the combination of flavonoids and their respective metabolites (quercetin/Q3G and hesperidin/hesperetin) were also associated with statistically significant increase in GSH levels (Fig. 1C and 1F). Overall these data indicate that flavonoids play an important role in the restoration of HR-induced GSH:GSSG ratio imbalance and hence may reduce oxidative stress during early trophoblast implantation.

3.2. The effect of quercetin and its metabolite Q3G on HR-induced p38 MAPK and JNK activation

We previously reported that flavonoids significantly reduced the activities of caspase 3/7 in the presence of HR [19]. Therefore, in this study we assessed the effect of flavonoids on MAPK pro-apoptotic pathways. We examined the activation of p38 MAPK and JNK pathways that are associated with pro-apoptosis by measuring the levels of activated p38 MAPK and JNK using phospho-specific antibodies. As shown in Fig. 2, HR-induced oxidative stress markedly

activated p38 MAPK and JNK phosphorylation in HTR-8/SVneo cells. We found that 24 h pre-treatment with quercetin (3 μM) was associated with significant inhibition of p38 MAPK phosphorylation when compared to HR cells (Fig. 2A). Similarly, pre-treatment with quercetin also markedly inhibited HR-induced JNK phosphorylation when compared to HR cells (Fig. 2D). We also found that HTR-8/SVneo cells pre-treated with Q3G was associated in significant inhibition of p38 MAPK (Fig. 2B) and JNK (Fig. 2E) phosphorylation. Further investigation with the combination of quercetin and Q3G (Fig. 2C) were associated with complete inhibition of p38 MAPK phosphorylation as opposed to quercetin alone. Similarly, this combination also inhibited JNK phosphorylation (Fig. 2F). Finally, the data from Fig 2, showed that there was no significant modulation of total p38 MAPK or JNK expression by HR, quercetin, Q3G or in combination. Overall these results indicate that the involvement of quercetin and its metabolite Q3G in the inhibition of p38 MAPK and JNK phosphorylation could be one of many mechanisms in flavonoid-mediated cytoprotection.

3.3. The effect of hesperidin and its metabolite hesperetin on p38 MAPK and JNK phosphorylation

We next investigated the effects of pre-treatment with hesperidin, its metabolite hesperetin alone or in combination on HR-induced p38 MAPK and JNK activation in HTR-8/SVneo cells. Pre-treatment (24 h) with 3 μM hesperidin was involved in the inhibition of p38 MAPK phosphorylation (Fig. 3A) and JNK phosphorylation (Fig. 3D) when compared to HR cells. In addition, we found that 1 μM hesperetin markedly inhibited the activation of p38 MAPK (Fig. 3B) and JNK (Fig. 3E). The combination of hesperidin and hesperetin was also associated in attenuating the activation of p38 MAPK and JNK (Fig. 3C and Fig 3F, respectively). These data may indicate that hesperidin, hesperetin or hesperdin and hesperitin in combination are involved in the inhibition of pro-apoptotic JNK and p38 MAPK pathways in HTR-8/SVneo

cells. Hence, suggesting that flavonoids may prevent oxidative stress-induced apoptosis during early pregnancy and therefore prevent shallow trophoblast invasion.

3.4. Pre-treatment with flavonoids significantly promotes HTR-8/SVneo cell invasion

To validate the role of flavonoids on trophoblast cell invasion under oxidative stress conditions, we pre-treated HTR-8/SVneo cells with flavonoids prior to HR-induced oxidative stress. As expected, a significantly reduced number of cells invaded following hypoxia-reoxygenation insult (Fig. 4A and Fig. 4C). Interestingly, pre-treatment with quercetin or Q3G alone were associated with significant increase in HTR-8/SVneo cell invasion when compared to HR conditions (Fig. 4A and Fig. 4C). We also found that the combination of quercetin and Q3G was also associated with a significant increase in the number of cells invaded in comparison to HR induced, as well as normoxic control cells (Fig 4A and Fig 4C).

Similarly, pre-treatment with 3 µM hesperidin and its metabolite, hesperetin (1 µM) were also associated with markedly enhancing HTR-8/SVneo cell invasion as seen in Fig. 4B and Fig 4D when compared to cells exposed to HR alone. Further investigation revealed that a combination of hesperidin and hesperetin was associated with significant increase in HTR-8/SVneo cell invasion when compared to HR condition as well as normoxic control cells (Fig. 4C and Fig. 4D). Overall, these data suggest that the involvement of flavonoids may play a crucial role in promoting trophoblast cell invasion under conditions of oxidative stress. This is of scientific relevance since shallow trophoblast invasion has been associated with pregnancy complications [8].

3.5. Quercetin and its metabolite Q3G promote growth of spheroids derived from HTR-8/SVneo cells

The beneficial effects of flavonoids/metabolites on HTR-8/SVneo invasion prompted us to investigate their effects on spheroid formation/growth and invasion. To achieve this aim,

spheroids were generated from HTR-8/SVneo cells (with and without flavonoid treatment prior to HR) using the soft-agar colony methodology. The data from Fig. 5A and Fig 5B show that HR insult significantly reduced spheroid formation as well as the growth area when compared to normoxic control. However, pre-treatment with quercetin, Q3G or quercetin/Q3G combination was significantly associated with an increase in spheroid formation/growth when compared to HR cells (Fig. 5A and Fig. 5B). We did not find any significant difference between flavonoid pre-treated cells and normoxic control cells. Herein, we have established that quercetin, Q3G alone or in combination significantly enhanced HTR-8/SVneo derived spheroidal stem cell generation and invasion.

3.6. Quercetin, hesperidin and their metabolites enhance HTR-8/SVneo cell spheroid invasion

The effect of flavonoids on the invasiveness of the spheroids generated from HTR-8/SVneo cells following HR-induced oxidative stress was also investigated. As shown in Fig. 6A and Fig. 6C, HR insult significantly inhibited the spheroid invasion when compared to control cells. Pre-treatment with quercetin and its metabolite Q3G was associated with a significant increase in the invasion of spheroids (Fig. 6A and Fig. 6C). There was no statistical difference between quercetin or Q3G pre-treated cells and their controls. Similar data were achieved following pre-treatment with hesperidin and hesperetin alone, which were significantly associated with an increase in the invasive capacity of spheroids generated from HTR-8/SVneo cells when compared to the invasive capacity of spheroids produced under HR conditions (Fig. 6B and Fig. 6D). Again, we showed that hesperidin or hesperetin play a significant role in HTR-8/SVneo spheroidal stem cell growth and invasion

Discussion

Dietary flavonoids play a significant role in the reduction of oxidative stress in HTR-8/SVneo cells by increasing cell viability, decreasing GSSG levels, reducing NADP/NADPH ratio, inhibiting caspase 3/7 activity and directly scavenging H_2O_2 [19]. Flavonoids are a group of naturally occurring phenolic compounds with significant antioxidant properties ubiquitously distributed in plants [23,24]. Although, over 8000 flavonoids have been identified to date only a relatively small number have a dietary contribution [23]. The present study aimed to investigate the effects of pre-treating HTR-8/SVneo cells with flavonoids (quercetin and hesperidin), their respective metabolites (Q3G and hesperetin) alone or in combination (quercetin/Q3G or hesperidin/hesperetin) on HR-induced oxidative stress. Herein we investigated GSH levels, activation of p38 MAPK and JNK, *in vitro* invasion and spheroid growth/invasion.

As stated before in the introduction, we specifically selected quercetin and hesperidin (and their metabolites) because their abundance in fruits and vegetables that are craved by pregnant women (17, 18). Selection was also based on their chemical configurations such as the presence or absence of functional group (hydroxyl) in the C-3 position of the C-ring (3-hydroxy and 3-desoxyflavonoids respectively) [23]. Quercetin belongs to the 3-hydroxyflavonoid class and flavonol subclass, it is the most common and studied flavonoid (over 3000 citations on PubMed) while hesperidin is classified as the 3-desoxyflavonoid class and flavanones subclass, which have not gained much interest like that of flavonols [25]. Flavonoid's antioxidant properties have been attributed to be highly dependent upon their structural configurations, especially the availability of their hydroxyl group [26]. Another unique feature of this current study is that fact that cytoprotection was achieved using concentrations that are within

physiological range and within the daily recommended intake. In contrast to other studies [27–31], higher concentrations (20-500 µM) of flavonoids are used that cannot be achieved physiologically by the daily intake of fruits and vegetables.

We have demonstrated that flavonoids/metabolites significantly increase GSH levels against HR-induced GSH depletion in HTR-8/SVneo cells. These data was are in agreement with other studies that used non-trophoblast cells such as COS-1 fibroblast-like cells and HEp2 human laryngeal carcinoma cells [32,33]. Comparable data have also observed from *in vivo* studies stating that flavonoids significantly increase GSH levels as a protective mechanism against oxidative stress [34–37]. A flavonoid-mediated increase in GSH levels can be attributed to their ability to directly or indirectly scavenge free radicals by interacting/activating with other antioxidants and/or inhibition of cellular oxidases [38]. It is also observed that decreased GSH levels have been observed in patients with PE [39,40]. In fact, proteomic analysis of PE placentae concluded that glutathione metabolism (decrease in GSH and increase in GSSG) in placental tissues contributes to the pathogenesis [16]. However, to our knowledge, there are no previous studies on the protective effects of flavonoids against oxidative stress-induced reduction of GSH in placental tissue/cell lines.

The activation of MAPK signalling (p38 MAPK and JNK) has been observed in response to several extracellular stimuli such as severe hypoxia or HR [41,42]. p38 MAPK and JNK are pro-apoptotic members of the MAPK family that have been implicated in oxidative stress-induced cell death and DNA damage [43,44]. In this study, we investigated the effect of flavonoids and their metabolites on HR-induced p38 MAPK and JNK activation. Herein, HR-induced p38 MAPK and JNK activation was assessed by Western blotting using phospho-specific antibodies that recognise phosphorylated motifs within activated p38 MAPK (Thr^{180}/Tyr^{182}) and JNK (Thr^{183}/Tyr^{185}). As expected, HR stimulated a robust increase in p38 MAPK and JNK activation in HTR-8/SVneo cells. The data obtained from the western analysis suggest that flavonoid-mediated cytoprotection is

associated with inhibition of p38 MAPK and JNK. Other studies have also found that quercetin significantly inhibits p38 MAPK and JNK activation following high glucose-induced oxidative stress in retinal ganglion (RGC-5) cells [45,46]. Likewise studies have shown that hesperidin attenuates apoptosis by inhibiting p38 MAPK and JNK activation triggered by high glucose in RGC-5 cells and human umbilical vein endothelial cells [47,48]. It was also notable that the activation of these signalling pathways during placental oxidative stress results in shallow trophoblast invasion and placental insufficiency [8,49,50]. Therefore, the data shown in this present study clearly shows these flavonoids inhibit p38 MAPK and JNK activation. In future, it may be useful for clinicians/midwifes to formulate a diet rich in these flavonoids during pregnancy to minimise the chances of oxidative stress.

A critical component for a successful pregnancy and healthy placental function is the proper development of angiogenic (adequate trophoblast invasion) and vascular (maternal spiral artery remodelling) networks [1,14]. Oxidative stress (such as hypoxia and/or hypoxia reoxygenation) during gestation results in impairment of trophoblast invasion and abnormalities in human placenta [14,49,51]. In this present study, we confirmed that exposure of HTR-8/SVneo cells to HR-induced oxidative stress significantly reduced trophoblast cell invasion. These data are in agreement with other studies using HR insult as a model to assess the invasiveness of HTR-8/SVneo and trophoblast primary cells [52–56]. However, this is the first report to show that pre-treatment with flavonoids/metabolites prior to oxidative stress insult significantly enhances trophoblast (HTR-8/SVneo) cell invasion. Although the exact mechanism behind flavonoid-mediated enhancement of invasion is still unknown, it may be possible that the antioxidative properties of flavonoids such as their ability to interact with/activate other antioxidative enzymes or the inhibition of JNK and p38 MAPK signalling contributes to this protection. Flavonoids may also be able to interact with key trophoblast regulators such as Notch

signalling, WNT signalling, VEGF family and TGF-β superfamily to ensure adequate trophoblast invasion and successful maternal spiral artery remodelling. Clearly further studies are required to investigate the potential modulation of these key trophoblast regulators by dietary flavonoids and their metabolites. In future, it may be possible to carry out knock-down experiments inhibiting this pathway would conform the role of these pathways in flavonoid induced cytoprotection.

The ability of HTR-8/SVneo cells to develop spheroidal stem cells has already been established in our group [57]. The use of spheroid cells embedded in matrix has been found to be an invaluable tool in analysing cell invasion due to their resemblance to trophoblasts. Also, using this methodology, the invasiveness of EVT cells can easily be measured to evaluate biological or pharmaceutical effects [58]. Herein, we investigated the effects of oxidative stress on the ability of HTR-8/SVneo cells to generate spheroidal stem cells and their subsequent invasive capacity. Initial studies revealed that HR-induced oxidative stress attenuated the ability of HTR-8/SVneo cells to generate spheroidal stem cells and their invasive capacity. The data revealed that quercetin and Q3G (or in combination) significantly enhanced spheroid generation and the growth area of spheroids, suggesting that flavonoids and their metabolites play an important role in HTR-8/SVneo derived stem cell survival against oxidative stress. During early gestation, the cytotrophoblast stem cells differentiate into the cytotrophoblast and synctiotrophoblasts, with the former giving rise to the invasive extravillous trophoblast cells that are responsible for maternal spiral conversion [6,59–61]. It has been reported that oxidative stress markedly affects stem cell self-renewal and their ability to differentiate into multiple cell types [62,63]. Our findings suggest that flavonoids and their metabolites may play a crucial role in the prevention of oxidative stress-induced impairment of the differentiation of cytotrophoblast

stem cells into trophoblast lineage. Since impairment of cytotrophoblast differentiation leads to poor placental development and pregnancy complications it is conceivable that dietary flavonoids may be of clinical benefit.

We subsequently investigated the invasive ability of spheroids generated from HTR-8/SVneo cells. Our data demonstrated that HR-induced oxidative stress significantly reduced spheroidal stem cell invasiveness. To the best of our knowledge, this is the first study to report that pre-treatment with quercetin, hesperidin and their metabolites (Q3G and hesperetin) significantly increased the invasive area of these spheroids, suggesting that flavonoids and their metabolites not only enhance spheroid generation and growth, but also enhance their invasiveness. The exact mechanism behind the effect on spheroid invasion remains unknown but may be attributed to the antioxidant properties of flavonoids as other antioxidant treatments have been demonstrated to increase proliferation, enhance mitochondrial integrity and suppress oxidative stress in human stem cells [64]. In addition, flavonoids have been proposed to enhance stem cell function by upregulating OCT4 gene expression and peroxisome proliferator-activated receptors (PPARs) [65,66]. Therefore, future studies should explore the effects of these flavonoids on other intracellular pathways such as Notch and WNT signalling during HR induced oxidative stress. It should be noted, that whilst the cytoprotective effects of quercetin and hesperidin were observed in this study at concentrations achievable via dietary intake, it is important to note that the beneficial effects observed in vitro may differ markedly from their in vivo effects due to bioavailability. This is particularly relevant to polyphenolic compounds such as flavonoids, which have poor bioavailability and as such may limit their therapeutic potential. The overall bioavailability of dietary flavonoids is complex and affected by several factors including chemical modifications via first-pass metabolism, low absorption in the

gastrointestinal tract, aqueous solubility, cell membrane permeability and in certain cases their ability to cross the blood brain barrier [67, 68]. Hence, caution is needed when translating in vitro effects into potential positive health benefits of dietary supplements.

It is also worth noting, in addition to causing apoptotic and necrotic cell death, oxidative stress can also trigger cellular senescence [69]. Higher levels of senescent cells may alter the growth properties and characteristics of HTR-8/SVneo cells due to the release of paracrine factors from senescent cells [70]. Indeed, it is interesting to note that cellular senescence plays a critical role in placental aging and abnormal senescence may play a role in pre-eclampsia [71]. Furthermore, a recent study has demonstrated that flavonoids, including quercetin, inhibited the expression of molecules associated with the development of the senescence-associated secretory phenotype in BJ fibroblasts [72]. Hence, it would be of great interest in future studies to explore in detail the effects of flavonoids on oxidative stress-induced cellular senescence in HTR-8/SVneo cells.

Overall, our results indicate that quercetin, hesperidin, and their metabolites alone or in combinations have a beneficial effect on trophoblast cell line against HR-induce oxidative stress by significantly increasing GSH levels and inhibiting p38 MAPK and JNK activation. Flavonoid treatment also enhanced trophoblast cell line invasion which is a major determinant of a successful pregnancy. Finally, flavonoid treatment increased trophoblast spheroid stem cell formation and invasion which are key factors in trophoblast differentiation during early gestation. The data from this study suggest that consumption of fruits and vegetable that are rich in quercetin or hesperidin may be beneficial to placental health during early gestation.

Author contribution

VJE has carried out the experiments and data analysis under the supervision of SDS and JMD. RMB developed the protocol for spheroid generation and invasion using HTR-8/SVneo cells and supervised VJE during spheroid growth/invasion experiments. Manuscript writing was collectively performed by all authors.

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Conflict of interest

None.

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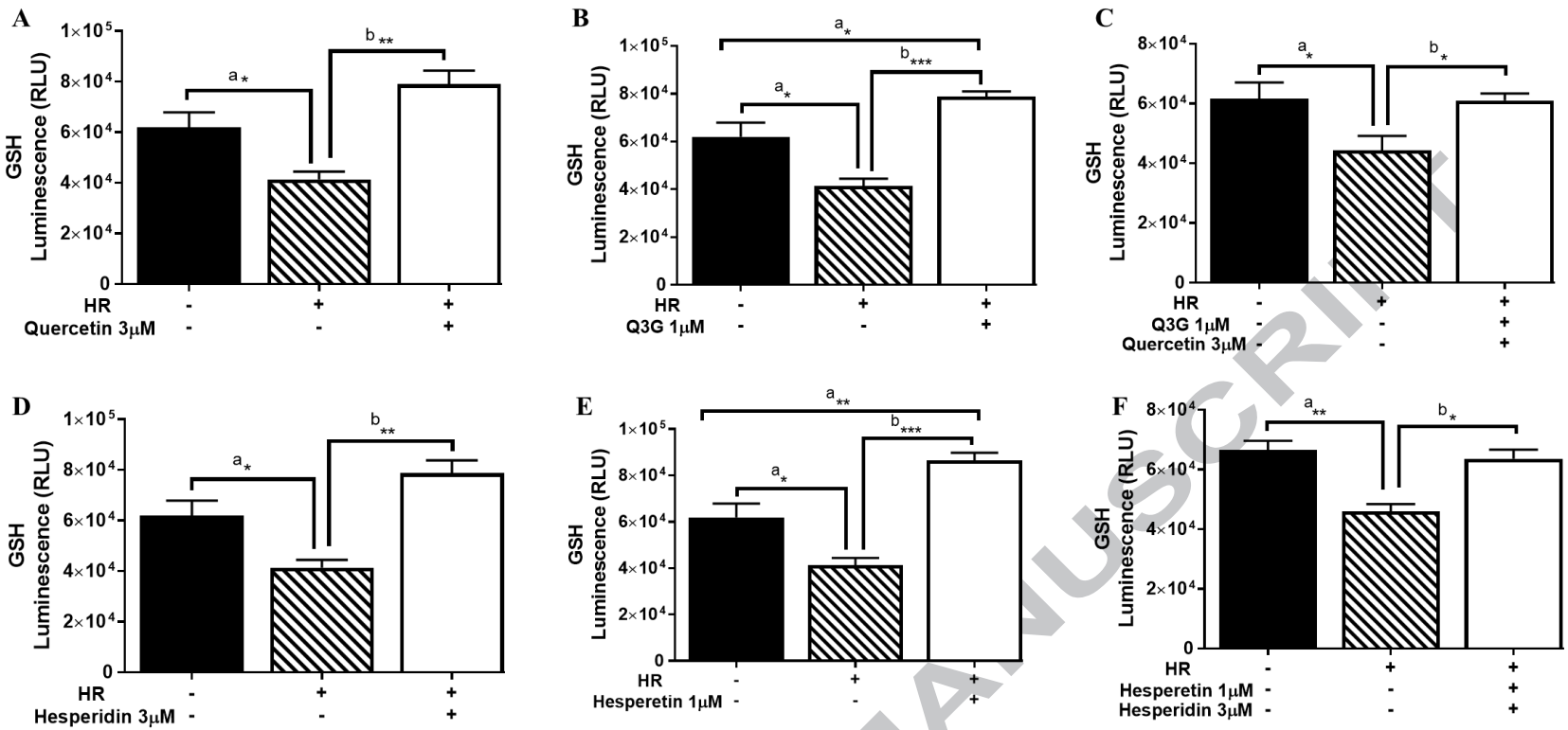


Figure 1: Effects of quercetin, hesperidin and their metabolites either alone or in combinations on glutathione in response to HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated concentrations of quercetin, hesperidin, their metabolites or combinations for 24 h prior to exposure to HR-induced oxidative stress. (A) quercetin, (B) Q3G, (C) quercetin/Q3G combination, (D) hesperidin, (E) hesperetin and (F) hesperidin/hesperetin combination. Data are expressed as the relative luminescence unit and represent the mean ± SEM of at least three independent experiments performed in triplicates. *P<0.05, **P<0.01, and ***P<0.001 (a) versus control and (b) versus HR.

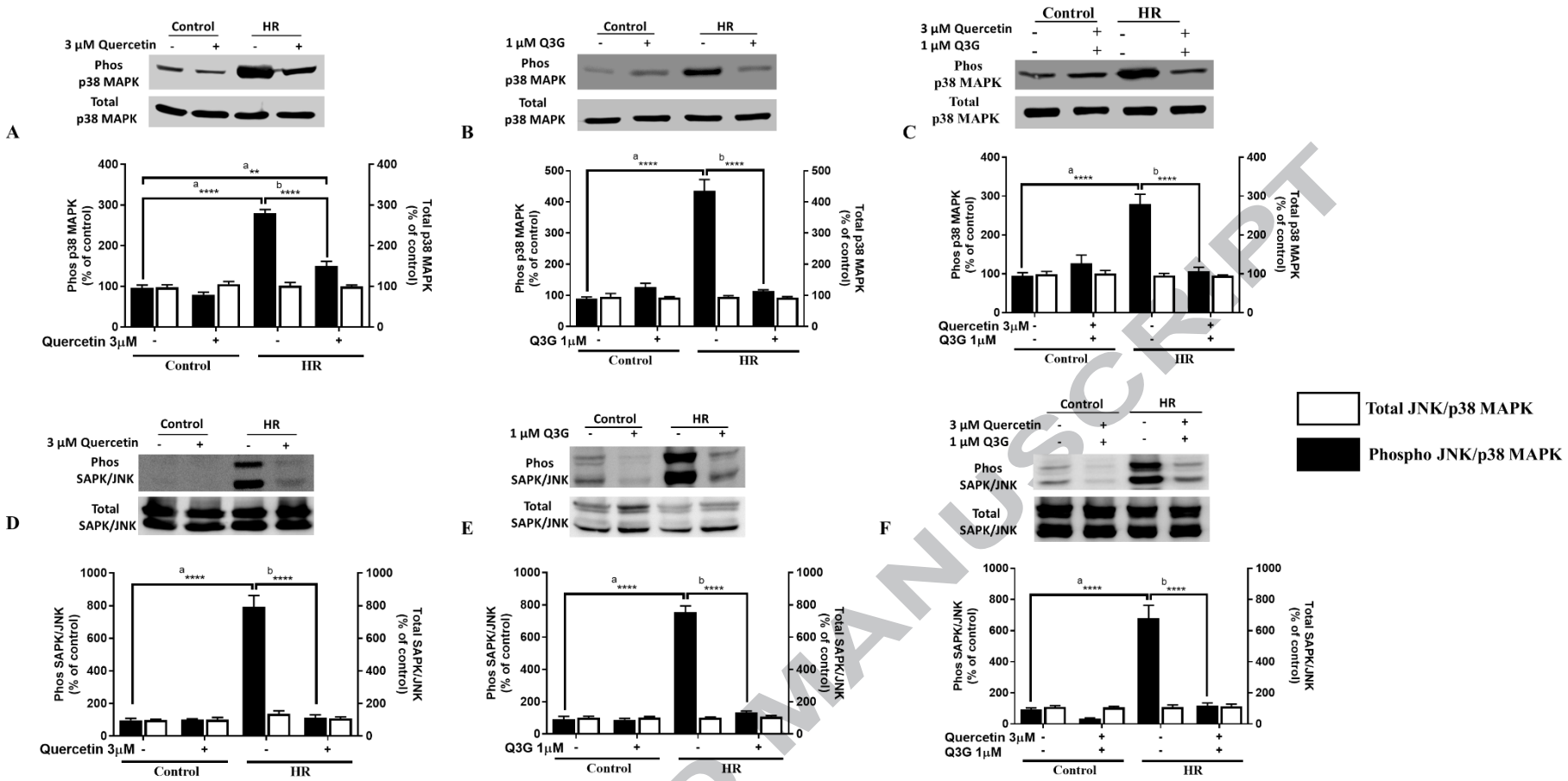


Figure 2: Effect of quercetin and its metabolite Q3G on HR-induced p38 MAPK and JNK activation in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated with 3 µM quercetin or 1 µM Q3G either alone or in combination for 24 h prior to HR-induced oxidative stress. Cell lysates were analysed by Western blotting for activation of p38 MAPK (A-C) and JNK (D-F) using phospho-specific antibodies. Samples were subsequently analysed on separate blots using antibodies that recognise total p38 MAPK and JNK. Data are expressed as the percentage of the value for control cells (=100%) in the absence of flavonoid and represent the mean ± SEM of three independent experiments. **P<0.01 and ****P<0.0001 (a) versus control and (b) versus HR.

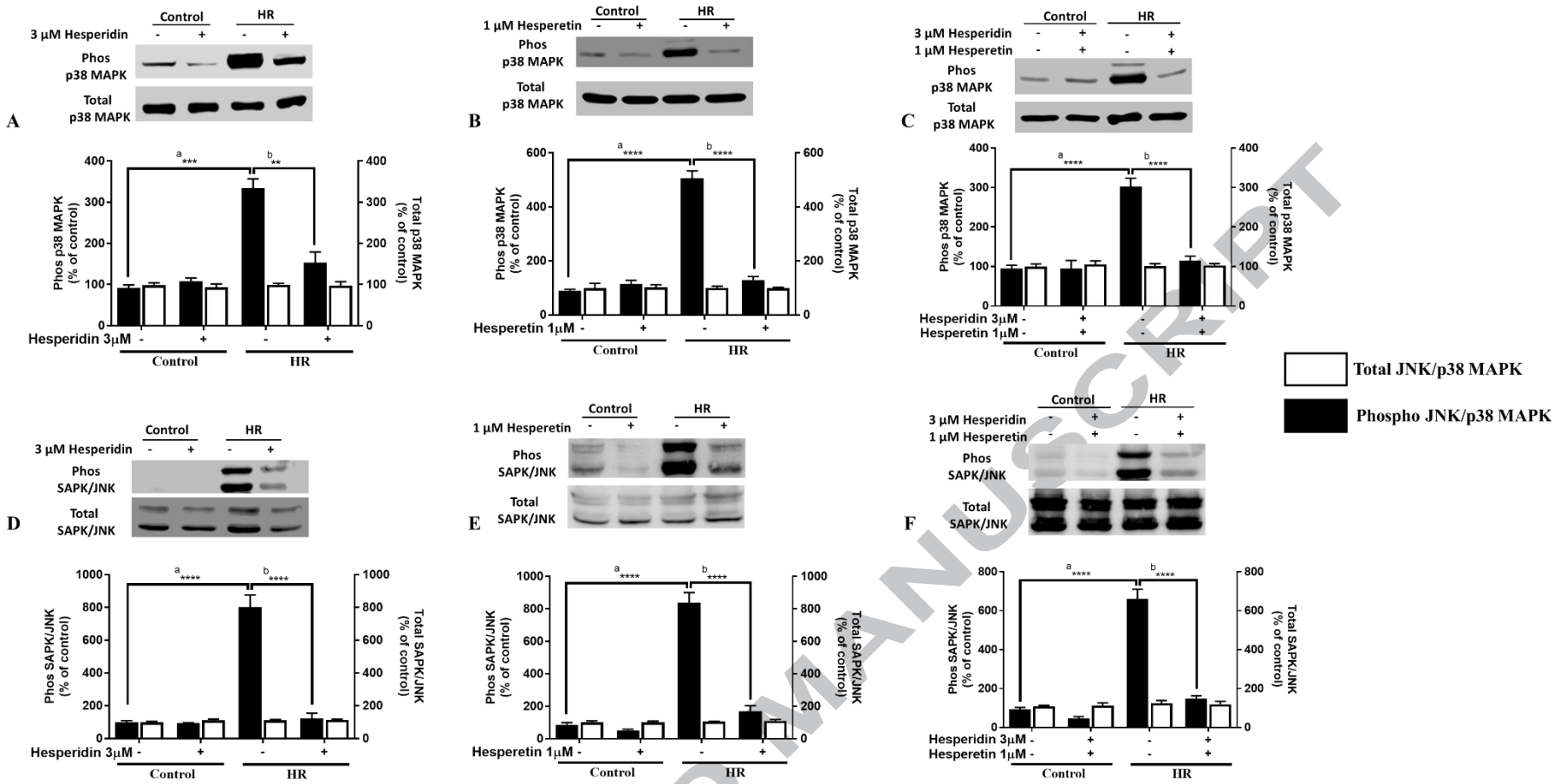
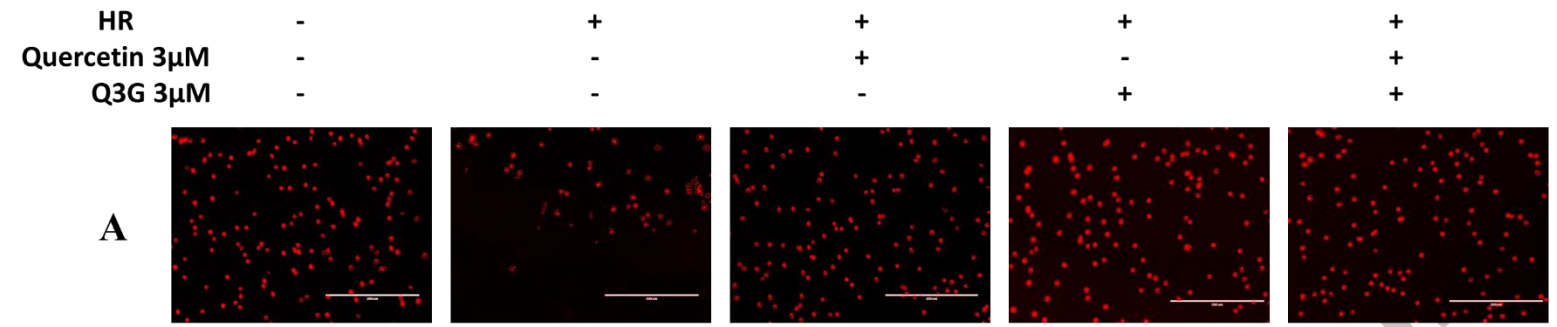


Figure 3: Effect of hesperidin and its metabolite hesperetin on HR-induced p38 MAPK and JNK activation in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated with 3 µM hesperidin or 1 µM hesperetin either alone or in combination for 24 h prior to HR-induced oxidative stress. Cell lysates were analysed by Western blotting for activation of p38 MAPK (A-C) and JNK (D-F) using phospho-specific antibodies. Samples were subsequently analysed on separate blots using antibodies that recognise total p38 MAPK and JNK. Data are expressed as the percentage of the value for control cells (=100%) in the absence of flavonoid and represent the mean ± SEM of three independent experiments. **P<0.01, ***P<0.001 and ****P<0.0001 (a) versus control and (b) versus HR.



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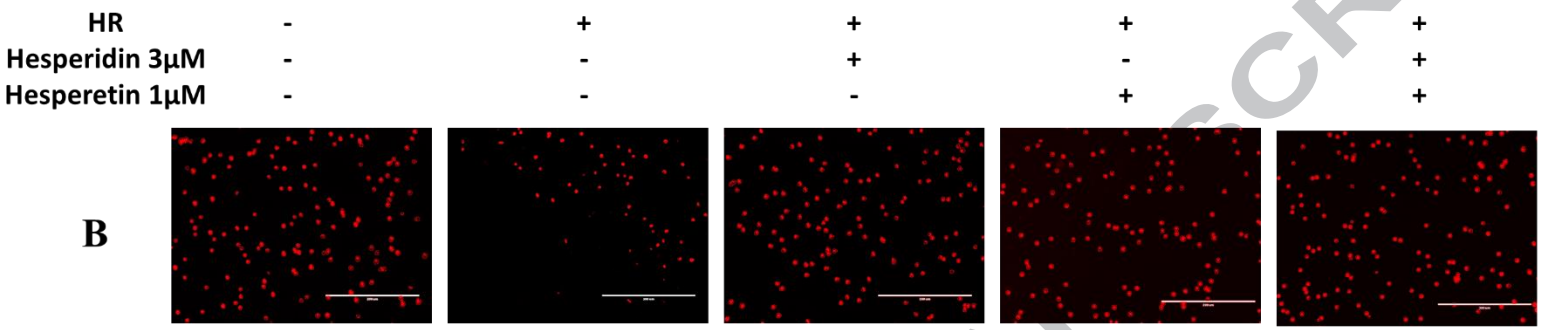

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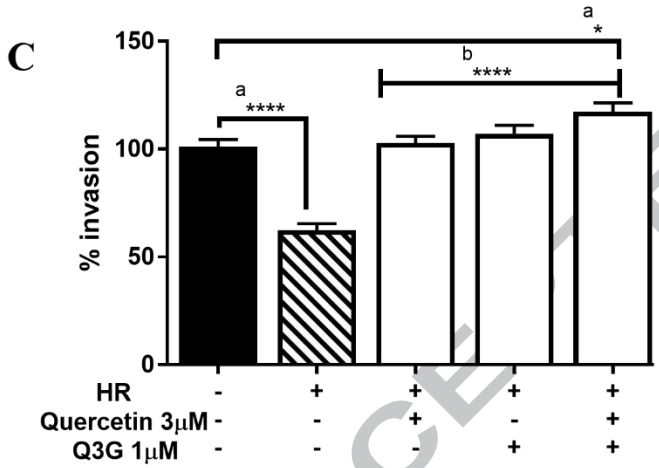

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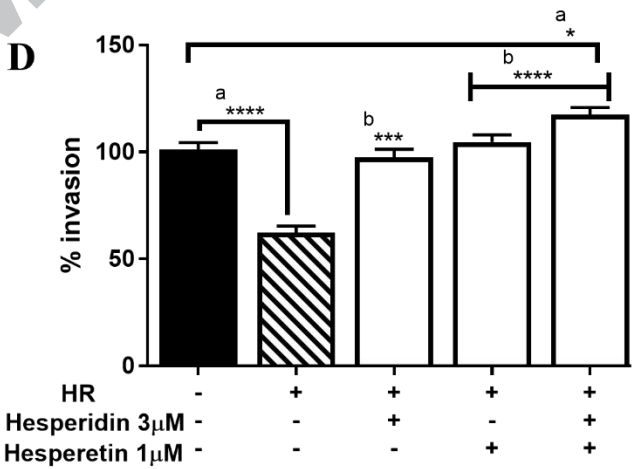


D

Figure 4: Effect of quercetin, hesperidin and their metabolites alone or in combination on HTR-8/SVneo cell invasion after HR-induced oxidation stress.

Cell invasion was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative images of cell invasion for control (untreated cells) and cells exposed to quercetin, Q3G alone or in combination for 24 h followed by HR, (B) Representative images of cell invasion for control (untreated cells) and cells exposed to hesperidin, hesperetin alone or in combination for 24 h followed by HR, Images were taken from the bottom coated membrane using a fluorescence microscope (EVOS FL Cell Imaging System) equipped with 20X objective magnification (Scale bar = 200 µm). (C and D) The percentage of invasion (number of cells invaded/number of cells migrated x 100) was performed using WimCounting (Cell Counting Image Analysis). Data are expressed as a percentage of control cells (non-HR) and represent the mean ± SEM of three independent experiments. *P<0.05, ***P<0.001 and ****P<0.0001 (a) versus control and (b) versus HR alone.

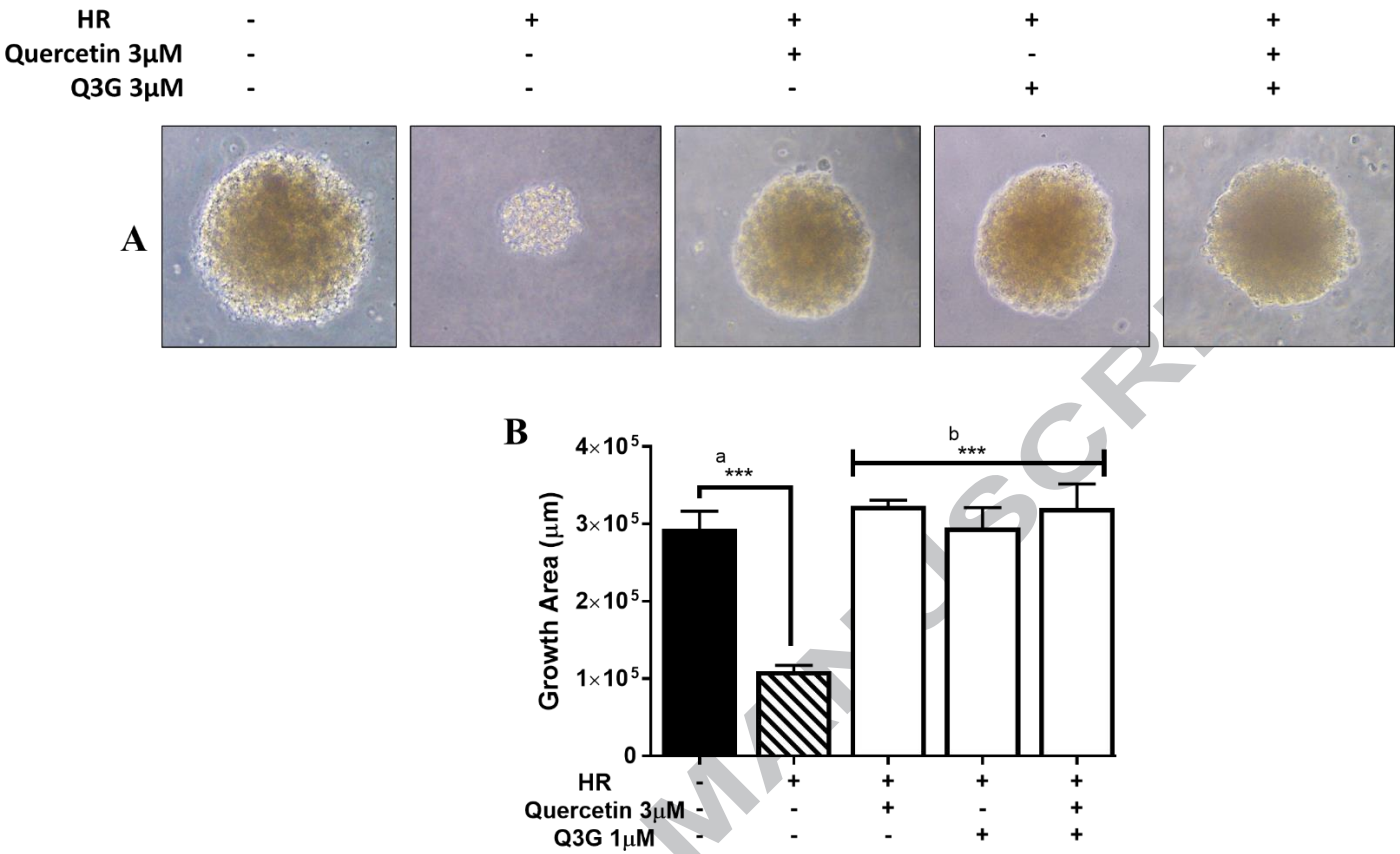
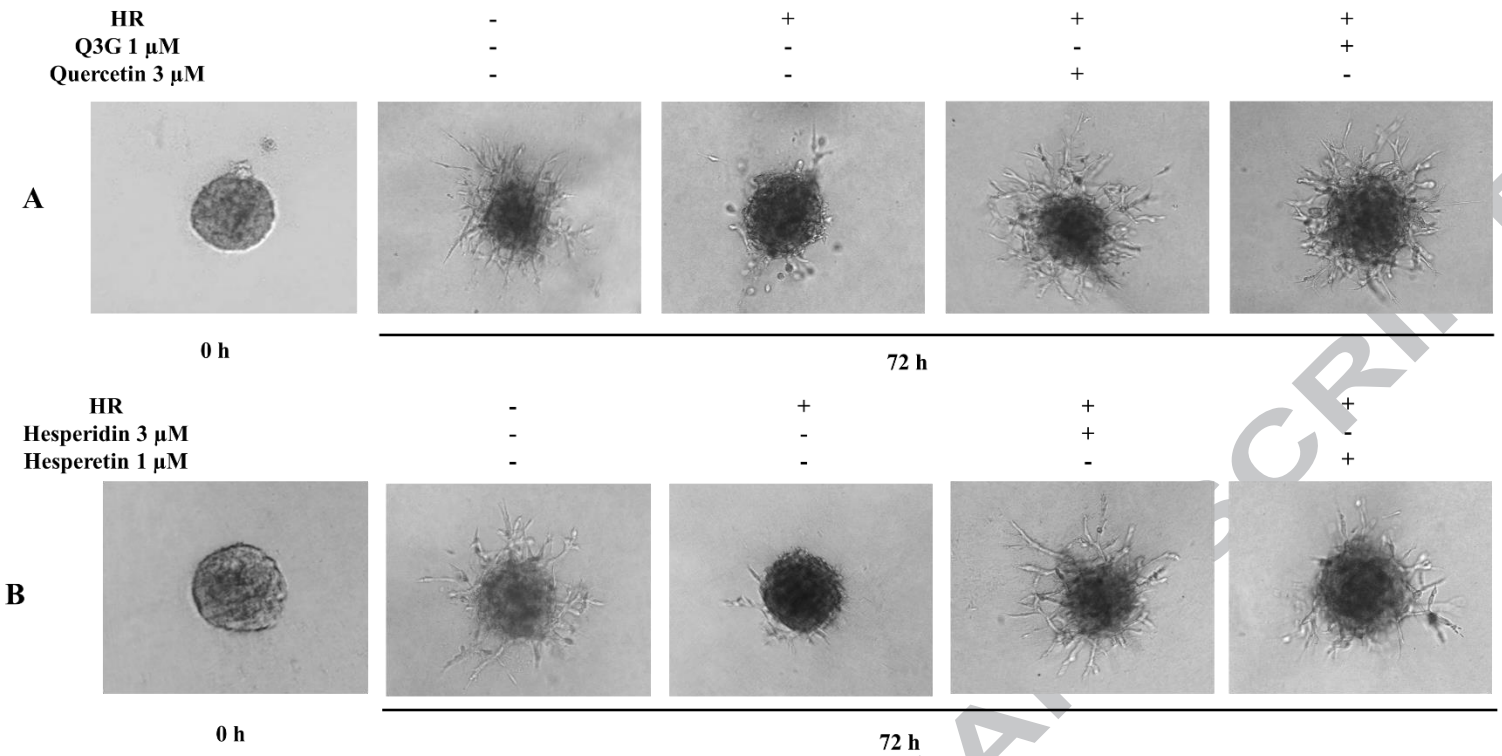
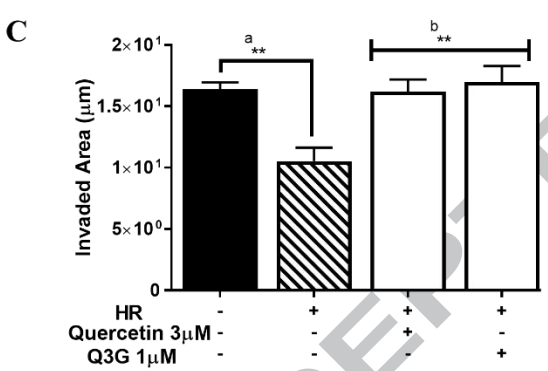


Figure 5: Effect of quercetin and its metabolite Q3G on the formation of spheroidal stem cells after HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated 3 µM quercetin and 3 µM Q3G either alone or in combination for 24 h prior to HR-induced oxidative stress. Cell suspensions (5 x 10^3 cells/well) were seeded in 6-well plates in 1% (w/v) agar and 0.7% (w/v) agarose mixture as described in Materials and Methods. (A) Representative images taken after 15 days using a Nikon Eclipse TS100 inverted microscopy attached with a Nikon DS-Fi2 camera with objective magnification 20X. (B) Quantitative analysis of growth area of the spheroids using WimColony (Cell Counting Image Analysis).

Data are expressed as area of cell growth (µm) and represent the mean ± SEM of three independent experiments. ***P<0.001 (a) versus control and (b) versus HR alone.





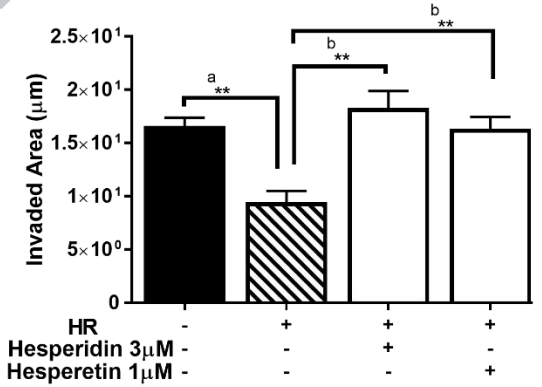


Figure 6: Quercetin, hesperidin and their respective metabolites enhance spheroid invasion after HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated flavonoids or metabolites for 24 h before exposure to HR-induced oxidative stress. Single cell suspensions were seeded at 3 x 10^3 cells/well in a 96 well 3D spheroid BME invasion plate (A and B) representative images taken at the start of the experiment (0 h; just after adding the spheroid invasion matrix) and subsequently following 72 h treatments as indicated. Images were taken using a Leica TCS SP5 II confocal microscope with objective magnification 10X. (C and D) Quantitative analysis of the spheroid invasion area was achieved using ImageJ software. Data are expressed as the area of cell invasion (µm) and represent the mean ± SEM of three independent experiments. **P<0.01 (a) versus control and (b) versus HR alone.

Figure Legend

Figure 1: Effects of quercetin, hesperidin and their metabolites either alone or in combinations on glutathione in response to HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated concentrations of quercetin, hesperidin, their metabolites or combinations for 24 h prior to exposure to HR-induced oxidative stress. (A) quercetin, (B) Q3G, (C) quercetin/Q3G combination, (D) hesperidin, (E) hesperetin and (F) hesperidin/hesperetin combination. Data are expressed as the relative luminescence unit and represent the mean ± SEM of at least three independent experiments performed in triplicates. *P<0.05, **P<0.01, and ***P<0.001 (a) versus control and (b) versus HR.

Figure 2: Effect of quercetin and its metabolite Q3G on HR-induced p38 MAPK and JNK activation in HTR-8/SVneo cells.

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