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Human pericyte-endothelial cell interactions in co-culture models mimicking the diabetic retinal microvascular environment.

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Abstract

Background: Pericytes regulate vascular tone, perfusion pressure and endothelial cell (EC) proliferation in capillaries. Thiamine and benfotiamine counteract high glucose-induced damage in vascular cells. We standardized two human retinal pericyte (HRP) / EC co-culture models, to mimic the diabetic retinal microvascular environment. We aimed at evaluating the interactions between co-cultured HRP and EC in terms of proliferation/apoptosis and the possible protective role of thiamine and benfotiamine against high glucose-induced damage.

Methods: EC and HRP were co-cultured in physiological glucose and stable or intermittent high glucose, with or without thiamine/benfotiamine. *No-contact model*: EC were plated on a porous membrane suspended into the medium and HRP on the bottom of the same well. *Cell-to-cell contact model*: EC and HRP were plated on the opposite sides of the same membrane. Proliferation (cell counts and DNA synthesis), apoptosis and tubule formation in Matrigel were assessed.

Results: In the no-contact model, stable high glucose reduced proliferation of co-cultured EC/HRP and EC alone and increased co-cultured EC/HRP apoptosis. In the contact model, both stable and intermittent high glucose reduced co-cultured EC/HRP proliferation and increased apoptosis. Stable high glucose had no effects on HRP in separate cultures. Both EC and HRP proliferated better when co-cultured. Thiamine and benfotiamine reversed high glucose induced-damage in all cases.

Conclusions: HRP are sensitive to soluble factors released by EC when cultured in high glucose conditions, as suggested by conditioned media assays. In the Matrigel models, addition of thiamine and benfotiamine re-established the high glucose-damaged interactions between EC/HRP and stabilized microtubules.

Keywords: human retinal pericytes, human endothelial cells, co-culture models, diabetic retinopathy, thiamine, benfotiamine.

Abbreviations: advanced glycation end product (AGE), benfotiamine (BT), bovine retinal pericytes (BRP), bromo-deoxy-uridine (BrdU), conditioned medium (CM), endothelial cells (EC), extracellular matrix (ECM), foetal calf serum (FCS), human retinal pericyte (HRP), high glucose (HG), intermittent high glucose (intHG), physiological glucose (NG), thiamine (T).

Introduction

Early diabetic retinopathy is characterized by thickening of the basement membrane and pericyte dropout. The latter has great consequences on capillary remodelling and may cause the first abnormalities that are observed clinically. Pericytes regulate vascular tone and perfusion pressure in capillaries and, being closely linked to endothelial cells, may modulate their proliferation [1]. Thickening of the basement membrane may interrupt this link and cause pericyte apoptosis and dropout, while endothelium, deprived of control, might proliferate in microaneurysms and, later, initiate angiogenesis [2]. Investigating the pathophysiological functions of endothelial cells and pericytes in an experimental contest where the interactions between these two cell types are preserved is therefore of great importance.

So far, most in vitro studies have been carried out with cells derived from retinas of animals that do not develop a retinopathy similar to that observed in humans with diabetes. The human retinal pericyte line (HRP) established in our laboratory [3] allows to obtain species-specific data. In fact, observations from our and other laboratories [4-6] suggest that human and bovine retinal pericytes (BRP) behave differently in experimental conditions designed to mimic the diabetic milieu. HRP are less prone to apoptosis induced by persistently high glucose than BRP. However, while BRP recover after returning to physiological levels, human pericytes are more vulnerable to both downwardly fluctuating glucose levels and intermittent exposure [5]. Apoptosis is strongly increased in HRP, but not in BRP, when they are cultured on extracellular matrix (ECM) produced by endothelium in high glucose conditions [6].

It has been repeatedly demonstrated that thiamine and its analogue benfotiamine have the potential to correct most of the known metabolic abnormalities induced by high glucose in isolated cells [7-9] and to prevent microangiopathy in animals with experimental diabetes [10-12]. Besides, initial evidence shows a potential role for this vitamin also in the prevention of nephropathy in diabetic subjects [13].

To gain further insight into the mechanisms regulating cell-to-cell interactions and activity of survival factors in an experimentally defined microenvironment, we have developed two co-culture models, using our immortalized HRP line and human endothelial cells (EC) to check the effects of hyperglycaemic-like microenvironments and the potential beneficial effects of thiamine and benfotiamine.

Materials and Methods

Cell cultures

EC were obtained from human umbilical cords, as previously described [14]. Pools of cells from 3-5 cords were grown in Medium 199 - Hepes Modification, added with 20% foetal calf serum (FCS), until confluent. In secondary cultures, EC were kept in M199 + 20% FCS + 50 µg/ml Endothelial Cell Growth Supplement. EC were characterized by von Willebrand factor indirect immunofluorescence.

HRP were immortalized in our lab, by selecting clones stably transfected to express Bmi-1, a transgene that increases telomerase activity, as previously described [3] and characterized by immunofluorescence staining for specific markers: α-smooth muscle actin, desmin, platelet derived growth factor-ß receptor and proteoglycan NG-2. They were grown in DMEM 5.6 mmol/l glucose with 20% FCS in primary cultures and 10% FCS in secondary cultures.

All reagents for cell cultures were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated.

Co-culture models

1. ***No-contact, bilayer shared-media model***. HRP (50,000/well) were plated onto the bottom surface of a 6-well plate, while EC (50,000/well, in order to have a ratio of EC/HRP of 1:1 similar to that of the human retina) were seeded on the inner surface of a 13-µm-thick, 25-mm-diameter polyethylene terephthalate (PET) membrane with 0.45-µm pores at a density of 1.6 million pores/cm^2 (Cyclopore membrane, Falcon Cell Culture Insert, Becton Dickinson, Franklin Lakes, NJ, USA), positioned above the HRP within the same well, without physical cell-to-cell contact. This creates a model in which the luminal surface of EC produces conditioned medium, to be shared with HRP (total volume 2.5 ml), at a separation distance of 0.9 mm (**Fig. 1a**). Control experiments were run, in which HRP and EC were plated on the relevant surfaces (well bottom or membrane) alone (i.e. without the other cell type). To understand the influence on HRP of soluble factors released by the endothelium, EC in the insert were kept for 8 days in physiological glucose (5.6 mmol/l, NG), high glucose (28 mmol/l, HG) or HG plus 50 µmol/l thiamine (HG+T) or benfotiamine (HG+BT), while HRP at the bottom of the well were kept in control physiological conditions. In a subsequent series of experiments, EC in the insert were exposed also to intermittent HG (intHG, 48 hrs NG/48hrs HG twice). Media were changed every 48 hrs in all groups (**Fig. 1b**). At the end of the incubation period, EC-containing inserts were

removed and put inside a new plate (**Fig. 1c**). EC and HRP were collected separately by trypsinization (**Fig. 1d**).

2. ***Cell-to-cell contact bilayer model***. In this model, HRP were plated onto the outer surface of the cell membrane, by turning the insert upside down. After they attached (2 hrs), the insert was capsized and relocated inside the well, and EC were plated on the opposite inner surface of the same membrane. This creates a model in which HRP are able to contact the abluminal surface of the EC through the pores of the membrane (**Fig. 1e**). Control experiments were run, as described above.

   EC were kept for 8 days in NG, HG, HG+T, intHG, intHG+T, intHG+BT, while HRP were grown in physiological conditions, as described above (**Fig. 1f**). At the end of the incubation period, trypsin-EDTA was added inside the insert to detach and collect EC, and inside the well at a volume sufficient to wet the bottom of the insert, in order to detach HRP, which are then collected from inside the well (**Fig. 1 g-h**).

3. ***Three dimensional co-culture in Matrigel***

   3D co-cultures were established by coating 24-well plates on ice with a mixture 1:1 of Matrigel™ (BD Biosciences, San Jose, California, USA) and M199 + 0.25% bovine serum albumin. This polymerizes in 1 hr at 37°C.

   50,000 HRP and EC, both pre-incubated separately for 8 days with NG and stable HG in a first series of experiment, and subsequently in NG and intHG with or without T and BT in a second series, were stained with two different viable fluorescent dyes, according to the manufacturer's procedure. EC were stained with the red fluorescent dye PKH26 (Sigma) and HRP with the green fluorescent dye CFDA (Vybrant CFDA Cell Tracer Kit, Invitrogen, Carlsbad, CA, USA).

   After staining, cells were plated together onto Matrigel with a ratio 1:1 and incubated at 37°C, monitoring the microtubule formation every 30' at the light microscope.

   Images were taken under a fluorescent microscope (Leica DM 2000, objective HCX FL PLAN 20x/0.40, Leica Microsystems, Wetzlar, Germany), equipped with a Leica DFC 320 camera and Leica Application Suite V3 digital processing and analysis software.

Proliferation and Apoptosis measurements.

Proliferation was assessed by either cell counts after Trypan blue staining and bromo-deoxy-uridine (BrdU) incorporation into the DNA, according to the manufacturer's instructions (Cell Proliferation Assay, BrdU, Roche Applied Science, Mannheim, Germany).

Apoptosis, in terms of DNA fragmentation, was assessed by ELISA, according to instructions (Cell Death Detection ELISAplus, Roche).

Statistical analysis

Results are mean ± SD of 6 independent experiments, normalized against control (NG), unless otherwise stated. Statistical comparisons among groups were carried out by two-tailed Student's *t*-test for paired data or Wilcoxon's Signed Ranks test, as appropriate. Results were considered significant if the p-value was 0.05 or less.

Results

Comparison between EC/HRP co-cultured or in solo cultures in physiological conditions

In the no-contact model, results show that both cell types proliferated better in physiological conditions (NG) when kept in co-culture than when cultured alone (**Fig. 2**). In particular, we observed a 25.7% reduction in the number of EC and a 26.1% reduction of HRP grown alone in comparison to the same co-cultured cell types ($p<0.05$) (**Fig. 2a**). DNA synthesis was reduced by 34.1% in EC and by 23.3% in HRP cultured alone in comparison with co-cultured EC/HRP ($p<0.05$) (**Fig. 2b**).

These findings were confirmed in the cell-to-cell contact model, with a 24.5% reduction in number of EC and 24.4% in HRP cultured alone ($p<0.05$ vs co-cultured cells, **Fig. 2c**). DNA synthesis decreased (-69.5% in EC and - 23.4% in HRP) in solo-cultures vs co-cultures ($p<0.05$, **Fig. 2d**).

Effects of high glucose and thiamine/benfotiamine on the no-contact co-culture model

To understand the possible influence on HRP of soluble factors released by the endothelium in diabetic-like conditions, we exposed EC in the insert first to stable HG and to HG plus thiamine or benfotiamine, and then to intHG also, while HRP at the bottom of the well were kept in physiological conditions, to mimic what happens in vivo, where endothelium is the first cell type coming into direct contact with hyperglycaemia. In control experiments EC and HRP were cultured separately and both exposed to the different experimental conditions.

High glucose concentrations reduced proliferation (both counts and DNA synthesis) and enhanced apoptosis in EC and HRP in co-cultures, and in EC alone, while it had no effect when HRP were cultured alone, similarly to our previous observations [5] demonstrating that human pericytes alone are not affected by stable high glucose conditions.

With reference to cell counts, co-cultured EC and HRP number decreased respectively by 22.3% and 26.1% in comparison to NG ($p<0.05$) (**Fig. 3 a,b**). EC in solo cultures decreased by 22.1% vs NG ($p<0.05$) (**Fig. 3a**).

DNA synthesis in co-cultured EC (**Fig. 3c**) and HRP (**Fig. 3d**) in HG showed a reduction of 21.2 and 14.3% respectively ($p<0.05$ vs NG), and 16.7% ($p<0.05$ vs NG) in EC cultured alone in HG (**Fig. 3c**).

DNA fragmentation, as a marker of apoptosis, was increased in co-cultured EC (+21.6%) and HRP (+53.6%), and in EC in monoculture (+17.1%) ($p<0.05$ vs NG) when kept in HG (**Fig. 4**).

Thiamine and benfotiamine were able to counteract the damaging effect of high glucose in all cases.

Intermittent high glucose exposure of EC, in separate experiments, had no significant effect on HRP proliferation/apoptosis (data not shown).

Effects of high glucose and thiamine/benfotiamine on the cell-to-cell contact bilayer model

High glucose reduced proliferation (both counts and DNA synthesis) and enhanced apoptosis in EC and HRP in cell-to-cell contact co-cultures, but had no effect when HRP were cultured alone. Intermittent high glucose was able to reduce proliferation and increase apoptosis in co-cultures, as well as HRP alone.

In terms of proliferation, co-cultured EC number was decreased by 25.2% in HG and 18.4% in intHG ($p<0.05$ vs NG) (**Fig.5a**) and HRP number by 28.3% and 37.2%, respectively, $p<0.005$ (**Fig. 5b**). HG had no effect on HRP cultured alone, while intHG reduced their number by 19.1% ($p<0.05$) (**Fig. 5b**).

DNA synthesis was reduced in the presence of both HG and intHG, in co-cultured EC (-27.1 and -24.2%, respectively, $p<0.05$ vs NG) and HRP (-15.8 and -14.0%, $p<0.05$) (**Fig. 5 c,d**).

DNA fragmentation increased in both HG and intHG, in co-cultured EC (+39.1% and +80.8%, respectively, $p<0.05$) and HRP (HG: +92.6%, intHG: +97.8%, $p<0.005$ vs NG) (**Fig. 6**). In HRP alone, intHG increased apoptosis by 13.9% ($p<0.05$) (**Fig. 6b**).

Thiamine and benfotiamine were able again to counteract the damaging effect of both stable and intermittent high glucose.

Three-dimensional co-culture in Matrigel

EC tend to rapidly form new capillaries when seeded into Matrigel, mimicking the later phases of angiogenesis [15]. We first checked if pre-treating both EC and HRP separately in stable NG vs HG for 8 days could have any effects on microtubule formation in Matrigel. Co-culturing pre-treated EC and HRP into Matrigel led to the formation of a capillary network in all the experimental

conditions; however, HG seemed to accelerate this process. In fact, after 3 hrs of culture in Matrigel, cells grown previously in HG have already organized in a network, in contrast with those kept in NG (**Fig. 7 a-h**). After 5 hrs in co-culture, microtubules in HG appear to be more disaggregate than in NG (**Fig. 7 i-r**).

Subsequently to these results, we pre-treated separately EC and HRP in NG, intHG, intHG+T, intHG+BT for 8 days and then co-cultured them in Matrigel for 4 hrs 30'. Staining with different fluorescent dyes allows one to evaluate the contribution of each cell type in microtubule formation. Analysis of merged fluorescent images of the same photographic field seems to reveal less interaction between EC and HRP in microtubule formation, if they had been cultured previously in intHG (**Fig. 8 e-h**), in comparison with those grown in NG (**Fig. 8 a-d**), confirming the results on proliferation (see above). Thiamine and benfotiamine, when added to intermittent HG, confirm their ability to normalize proliferation and, moreover, appear to re-establish the interactions between EC/HRP, thus stabilizing the microtubules (**Fig. 8 i-n, o-r**).

Osmotic controls

Measurements of glucose concentrations and pH in the media at different time-points did not show any relevant modification from the starting conditions (data not shown). Osmotic pressure after addition of HG was 329±6 vs 309±4 mosmol/l of NG; addition of T/BT had no effects. Equimolar addition of mannitol instead of glucose to the culture media had no relevant effects (data not shown).

Discussion

Most studies on pericyte structure and function have been carried out so far using bovine or rat cells, because of their easier availability. We have demonstrated great differences between bovine and human retinal pericytes cultured in diabetic-like conditions: human pericytes are less sensitive to apoptosis induced by steadily high glucose than BRP, but are vulnerable to intermittent exposure to high and physiological glucose [5] and to culture on ECM produced by EC cultured in high glucose conditions [6]. For this reason, an immortalized human retinal pericyte line, with extended proliferation capability and stable differentiation, together with an ability to be influenced by glucose toxicity completely super-imposable to the one of the primary cells from which it was derived [5], was established and characterized in our lab [3] and used for this study.

Previous studies on pericyte function, including their synthesis of ECM components and response to growth activators and inhibitors, have been conducted mostly on homogenous cultures. In vivo, however, pericytes live in close contact with endothelial cells, with which they interact tightly and

on which they exert several levels of control. Consequently, it is important to study their pathophysiological mechanisms in experimental contests where the interactions between these two cell types are preserved. Even though some studies in the literature used EC-pericyte co-cultures [16-18], in our knowledge this is the first time in which pericytes and EC of human origin have been utilized.

Our "no-contact" EC-HRP co-culture model allows studying the influence of soluble factors released in the shared medium by one cell type on the survival of the other, while the "contact" model permits the evaluation of any effect(s) due to direct interactions between the two cell types, rather than mediated through the culture medium. Evidence in the literature shows that smooth muscle cells, grown on the outer surface of the same type of membrane used in this work, send out cytoplasmic projections able to penetrate the pores and make intimate contact with EC [19] and the same membrane has been used to create other direct co-culture models [16].

Interactions between endothelium and pericytes are important for cell survival and proliferation, as supported by our data showing that both EC and HRP proliferate better when co-cultured in physiological conditions than alone. This is in contrast with some observations in the literature, showing that pericytes are able to inhibit EC proliferation when co-cultured in direct contact [17, 20]. However, those results were obtained with bovine or porcine cells, not human-derived cells. Species-specific differences in metabolism may play the difference and further underline the need for human models when studying human disease.

A direct correlation between hyperglycaemia and microvascular diabetic complications (in particular diabetic retinopathy) has been demonstrated by large cohort studies [21, 22]. In our models, both cell types reduced their proliferation and increased apoptosis when co-cultured in stable high glucose conditions. This happened also in EC cultured alone, consistently with previous evidence in the literature [23-25], while HRP in solo cultures showed neither reduced proliferation nor increased apoptosis, confirming our previous data on low sensitivity of human pericytes to persistently high glucose in comparison with bovine cells [5]. Exposure to intermittent high glucose in the contact model, on the contrary, resulted in decreased proliferation and increased apoptosis in all cases, including HRP alone, consistently with our previous findings that HRP are vulnerable to both downwardly fluctuating glucose levels and intermittent exposure [5]. There is evidence to suggest that rapid glucose depletion [26, 27] enhances apoptosis in bovine retinal pericytes, while intermittent high glucose exposure increases apoptosis in human retinal [28] and umbilical vein [29-30] EC and induces an over-production of reactive oxygen species (ROS) in porcine EC [31]. Clinical observations also show that daily fluctuations in plasma glucose concentrations, as they occur in diabetic patients, correlate with increased risk of cardiovascular disease [32] and

microvascular complications [33]. Yet, it is not clear why in the no-contact model the intermittent high glucose exposure of EC only does not influence pericyte survival/apoptosis: it is possible that the deleterious effect of intermittent HG on pericytes needed a tighter contact between the cells, as happens in vivo.

The importance of cell-to-cell communication through release of soluble factors, adhesion molecules, receptor-ligand interactions, mRNA-containing microvesicles, microRNA and organelles able to regulate protein expression and activity in target cells is well demonstrated in the literature [34-37]. The retinal vasculature is strictly regulated by EC-pericyte interactions, which include balanced production of positive and negative regulatory factors; therefore, the survival/loss of pericytes in diabetic retinopathy depends not only on their direct interactions with EC but also on soluble factors released by EC in hyperglycaemic conditions [34, 38-41]. Our data demonstrate that co-cultured, but not solo-cultured HRP are damaged by stable exposure to high glucose concentration, in terms of increased apoptosis and reduced proliferation. This suggests a mediation of endothelium, which in vivo is the first target of hyperglycaemia, but could filter its deleterious effects towards the underlying pericytes, a situation that we have tried to mimic in our co-culture models.

The impact of glucose toxicity on our retinal microvascular cell model was evident also on microtubule formation. EC and HRP, pre-cultured in high glucose and then seeded on Matrigel, began to create a tubular network earlier (3 hrs) than those pre-cultured in physiological conditions. After 5 hrs, however, this network was already disrupted, while it was still evident in physiological glucose pre-treated cells.

Finally, our results confirm once more the protective role of thiamine and benfotiamine, which were able to normalize proliferation and apoptosis in EC and HRP cultured in stable high glucose in the no-contact model and in intermittent high glucose in the contact model. In the Matrigel experiments, their beneficial effect is even more evident, as we observed reduced proliferation and a decrease in EC/HRP interaction after pre-treatment in intermittent high glucose, which were corrected by both thiamine and benfotiamine with consequent microtubule stabilization. The protective role of this vitamin against high glucose-induced metabolic damage in microvascular cells is now widely known [8, 9], as it reduces excess production of ROS, the common denominator of the four pathways involved in glucose damage (polyol pathway, AGE production, PKC activation and hexosamine pathway) [7, 42-43].

In conclusion, our results show that endothelial cells and pericytes interact strictly, influencing each other in terms of proliferation and survival, both directly, through cell-to-cell contact, and indirectly, through the secretion of soluble factors. However, from these experiments, it is not easy

to sort out which effects of co-culture are attributable to contact and which are due to the release of soluble factors. In fact, the cell-to-cell contact model does not exclude the influence of soluble factors released by the EC and the no-contact model seems to produce similar results, as compared with the cell-to-cell contact model.

Advances in the understanding of factors which influence endothelial cell and pericyte function and dysfunction yielding potentially exploitable therapeutic avenues would be of great importance. The validation of suitable co-culture models could be useful to test stimulatory and inhibitory agents, signals and drugs to avoid pericyte dropout and the development of diabetic retinopathy. The protective role of thiamine and benfotiamine on the retinal microvascular network in diabetic-like conditions is also confirmed, further strengthening the need to study the potential role of this vitamin in the prevention and/or treatment of diabetic microvascular complications.

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Conflicts of interest

The Authors declare no conflicts of interest.

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Figure legends

Fig. 1 Co-cultures experimental design. No-contact model: **a**) HRP were plated onto the bottom surface of a 6-well plate and EC on the membrane of an insert positioned above the HRP within the same well. **b**) To understand the influence on HRP of soluble factors released by the endothelium, EC in the insert were kept for 8 days in the relevant media, and HRP in control conditions. **c**) At the end of the incubation period, EC-containing inserts were removed and put inside a new well. **d**) EC and HRP were collected separately by trypsinization. **Cell-to-cell contact model**: **e**) HRP were plated onto the outer surface and EC on the opposite inner surface of the same membrane. **f**) EC in the insert were kept for 8 days in the relevant media, and HRP in control conditions. **g**-**h**) At the end of the incubation period, trypsin-EDTA was added inside the insert to detach and collect EC and inside the well to detach and collect HRP.

Fig. 2 Comparison between co-cultured EC/HRP vs in solo cultures in physiological conditions (NG). Mean of six experiments ± SD.
a,**c**) cell counts; **b**,**d**) DNA synthesis; **a**,**b**) no-contact co-culture model; **c**,**d**) cell-to-cell contact bylayer model. * = $p<0.05$ vs EC/HRP co-cultured in NG

Fig. 3 Proliferation of EC/HRP in the no-contact co-culture model: effects of high glucose and thiamine/benfotiamine. Mean of six experiments ± SD.
a) EC counts; **b**) HRP counts; **c**) EC DNA synthesis; **d**) HRP DNA synthesis. * = $p<0.05$ vs EC/HRP co-cultured in NG; # = $p<0.05$ vs EC/HRP co-cultured in HG, $ = $p<0.05$ vs EC grown alone in NG; & = $p<0.05$ vs EC grown alone in HG

Fig. 4 Apoptosis (DNA fragmentation) of EC/HRP in the no-contact co-culture model: effects of high glucose and thiamine/benfotiamine. Mean of six experiments ± SD.
a) EC apoptosis; **b**) HRP apoptosis. * = $p<0.05$ vs EC/HRP co-cultured in NG; # = $p<0.05$ vs EC/HRP co-cultured in HG, $ = $p<0.05$ vs EC grown alone in NG; & = $p<0.05$ vs EC grown alone in HG

Fig. 5 Proliferation of EC/HRP in the cell-to-cell contact bilayer model: effects of high glucose and thiamine/benfotiamine. Mean of six experiments ± SD.
a) EC counts; **b**) HRP counts; **c**) EC DNA synthesis; **d**) HRP DNA synthesis. * = $p<0.05$ vs EC/HRP co-cultured in NG; # = $p<0.05$ vs EC/HRP co-cultured in HG, & = $p<0.05$ vs EC/HRP co-

cultured in intHG; $ = p<0.05 vs EC/HRP grown alone in NG; £ = p<0.05 vs EC grown alone in HG or HRP grown alone in intHG

Fig. 6 Apoptosis (DNA fragmentation) of EC/HRP in the cell-to-cell contact bilayer model: effects of high glucose and thiamine/benfotiamine. Mean of six experiments ± SD.
a) EC apoptosis; **b**) HRP apoptosis.
* = p<0.05 vs EC/HRP co-cultured in NG; # = p<0.05 vs EC/HRP co-cultured in HG, & = p<0.05 vs EC/HRP co-cultured in intHG; $ = p<0.05 vs EC/HRP grown alone in NG; £ = p<0.05 vs EC grown alone in HG or HRP grown alone in intHG

Fig. 7 Effects of stable HG pre-treatment on microtubule formation in Matrigel. Co-culturing EC and HRP into Matrigel led to the formation of a capillary network. HG accelerates this process: after 3 hrs in Matrigel, stable HG pre-cultured cells have already organized in a network, in contrast with those kept in NG (**a-h**). After 5 hrs, microtubules in HG appear to be more disaggregate than in NG (**i-r**).
(**a, e, i, o**): microtubules at the phase contrast microscopy; (**b, f, l, p**): fluorescent images of the same microtubules showing Vybrant CFDA (green) – stained HRP, (**c, g, m, q**) PKH26 (red) – stained EC and (**d, h, n, r**) merge of the first 3 pictures. **a, b, c, d**: NG 3 hrs; **e, f, g, h**: HG 3 hrs; **i, l, m, n**: NG 5hrs; **o, p, q, r**: HG 5 hrs. Magnification bar: 50 µm

Fig. 8 Effects of intHG with or w/o T/BT pre-treatment on microtubule formation after 4hrs 30' of culture in Matrigel. Analysis of merged fluorescent images of the same photographic field reveals less interaction between intHG pre-treated EC and HRP in microtubule formation (**e-h**), in comparison with NG pre-treated ones (**a-d**). T and BT, when added to intHG, appear to re-establish their interactions, thus stabilizing the microtubules (**i-n, o-r**).
(**a, e, i, o**): microtubules at the phase contrast microscopy; (**b, f, l, p**): fluorescent images of the same microtubules showing Vybrant CFDA (green) – stained HRP, (**c, g, m, q**) PKH26 (red) – stained EC and (**d, h, n, r**) merge of the fluorescent images. **a, b, c, d**: NG; **e, f, g, h**: intHG**; i, l, m, n**: intHG + T; **o, p, q, r**: intHG + BT. Magnification bar: 50 µm

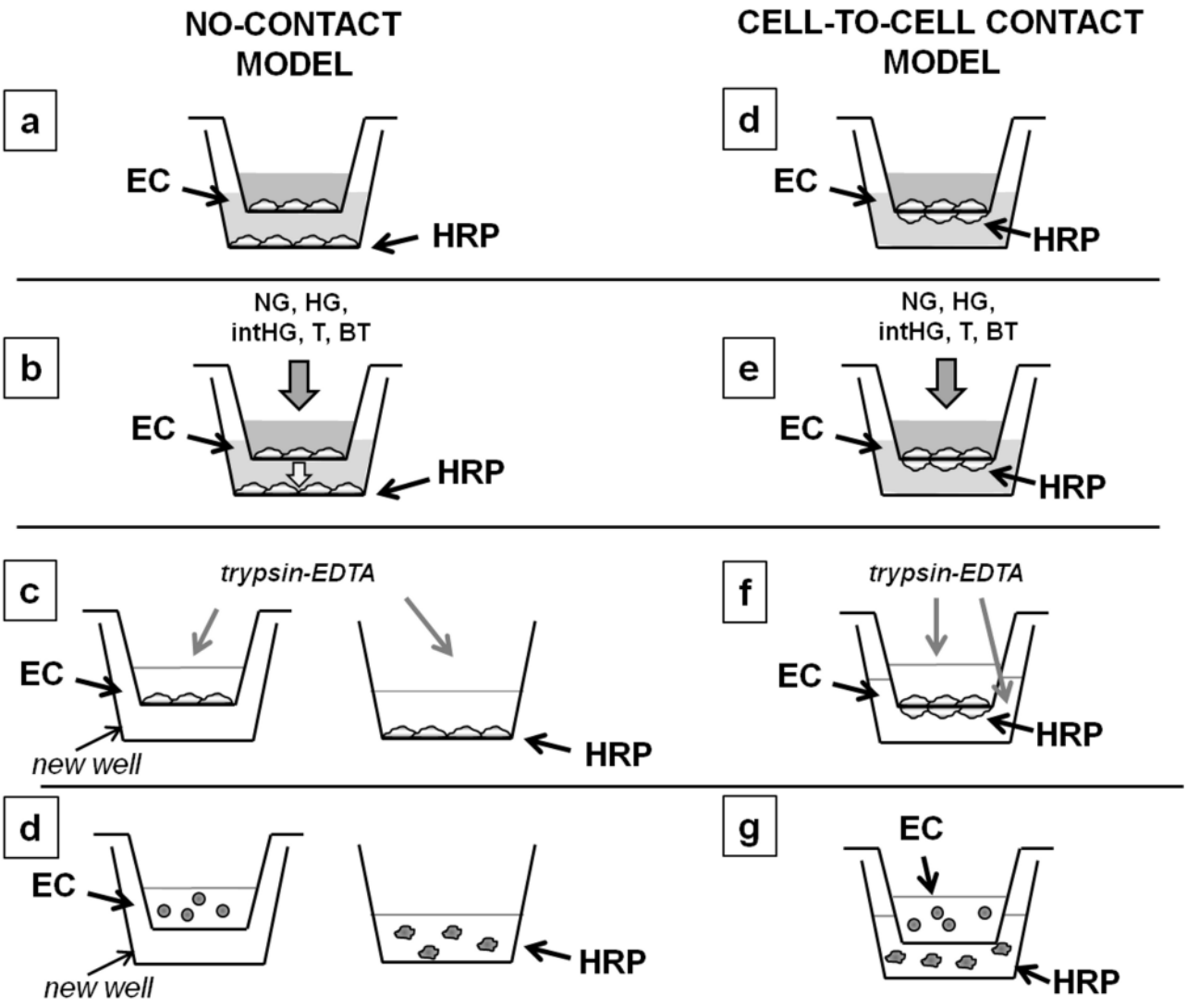
NO-CONTACT MODEL
CELL-TO-CELL CONTACT MODEL
a
EC
HRP
d
EC
HRP
NG, HG, intHG, T, BT
b
EC
HRP
NG, HG, intHG, T, BT
e
EC
HRP
trypsin-EDTA
c
EC
new well
HRP
trypsin-EDTA
f
EC
HRP
d
EC
new well
HRP
g
EC
HRP

Figure 1

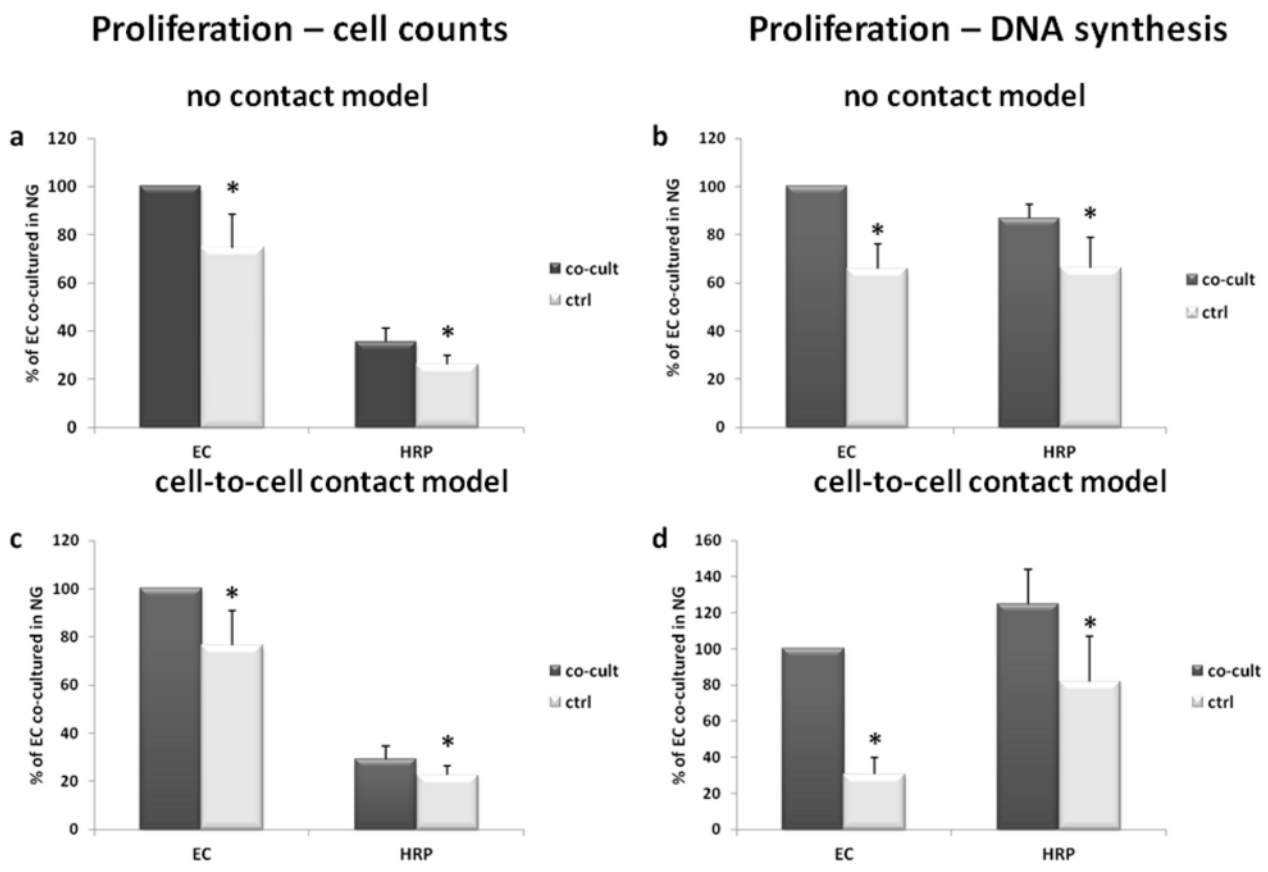
Proliferation – cell counts
Proliferation – DNA synthesis
no contact model
no contact model
a
b
% of EC co-cultured in NG
co-cult
ctrl
EC
HRP
cell-to-cell contact model
cell-to-cell contact model
c
d

Figure 2

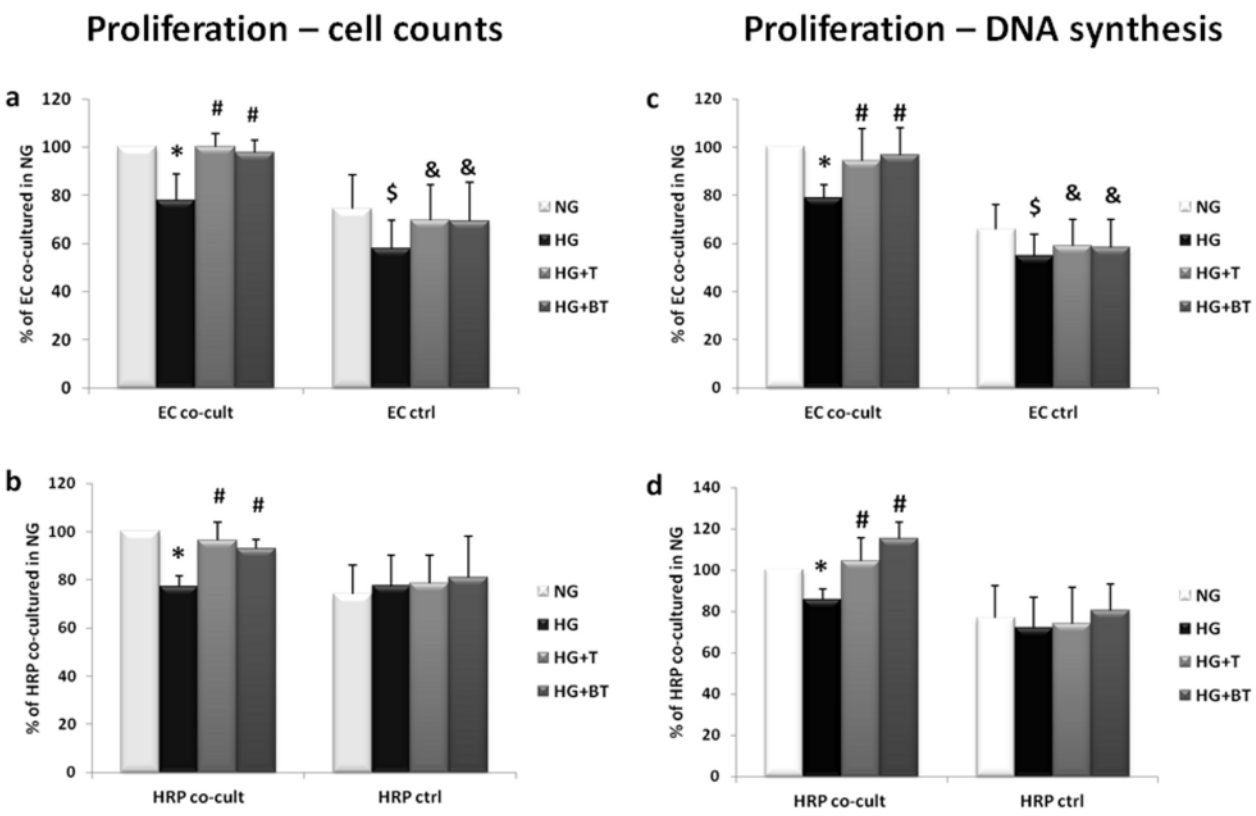
Proliferation – cell counts
Proliferation – DNA synthesis
a
b
c
d
% of EC co-cultured in NG
% of HRP co-cultured in NG
EC co-cult
EC ctrl
HRP co-cult
HRP ctrl
NG
HG
HG+T
HG+BT
*
#
$
&

Figure 3

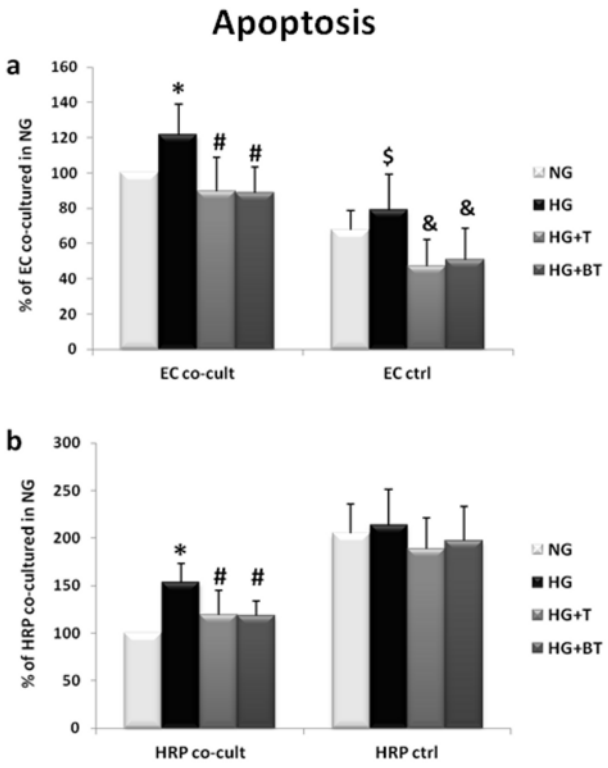
Apoptosis
a
% of EC co-cultured in NG
160
140
120
100
80
60
40
20
0
*
#
#
$
&
&
EC co-cult
EC ctrl
NG
HG
HG+T
HG+BT
b
% of HRP co-cultured in NG
300
250
200
150
100
50
0
*
#
#
HRP co-cult
HRP ctrl
NG
HG
HG+T
HG+BT

Figure 4

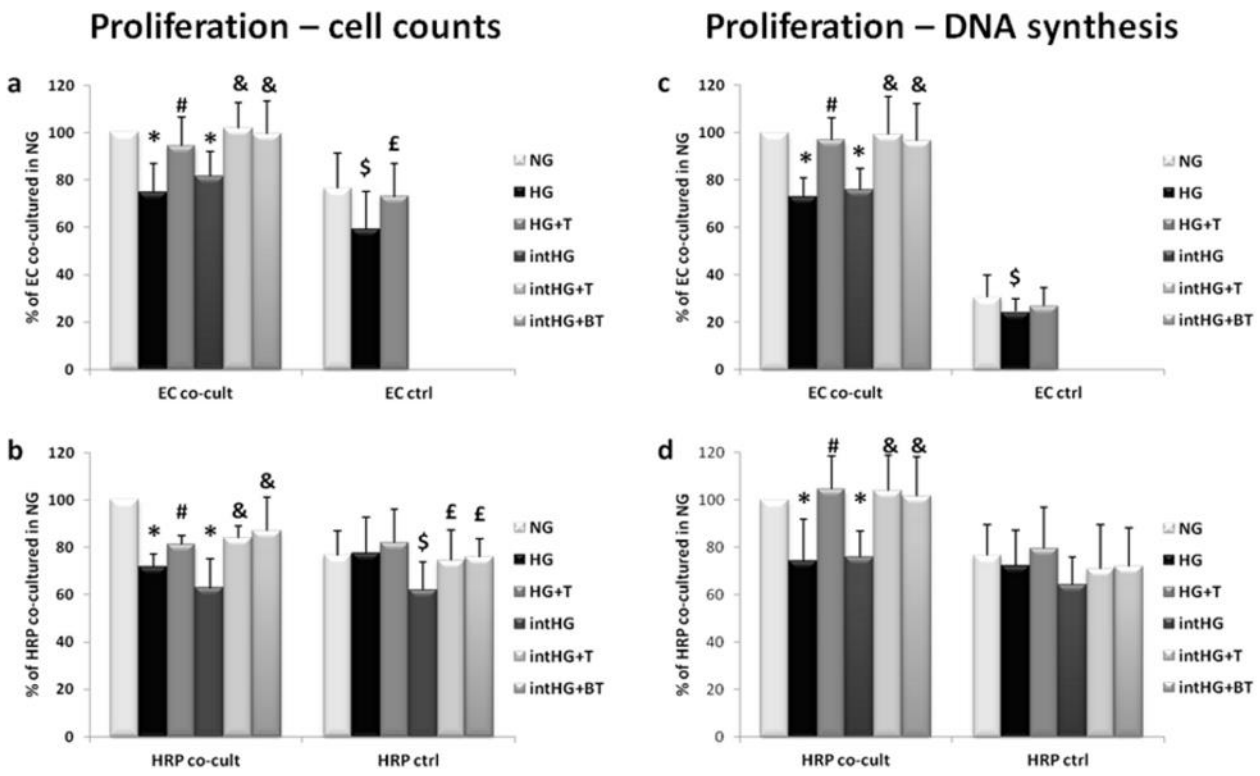
Proliferation – cell counts
Proliferation – DNA synthesis
a
b
c
d
% of EC co-cultured in NG
% of HRP co-cultured in NG
EC co-cult
EC ctrl
HRP co-cult
HRP ctrl
NG
HG
HG+T
intHG
intHG+T
intHG+BT

Figure 5

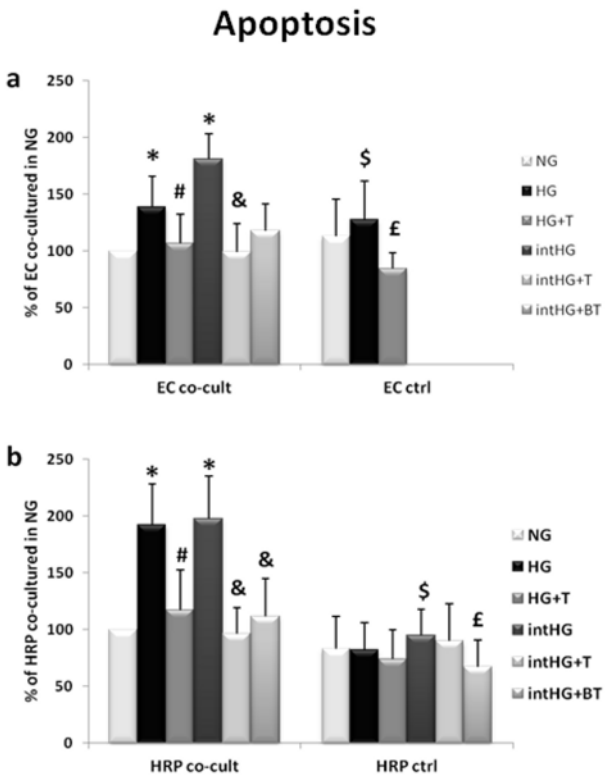
Apoptosis
a
% of EC co-cultured in NG
250
200
150
100
50
0
*
#
*
&
$
£
EC co-cult
EC ctrl
NG
HG
HG+T
intHG
intHG+T
intHG+BT
b
% of HRP co-cultured in NG
250
200
150
100
50
0
*
#
*
&
&
$
£
HRP co-cult
HRP ctrl
NG
HG
HG+T
intHG
intHG+T
intHG+BT

Figure 6

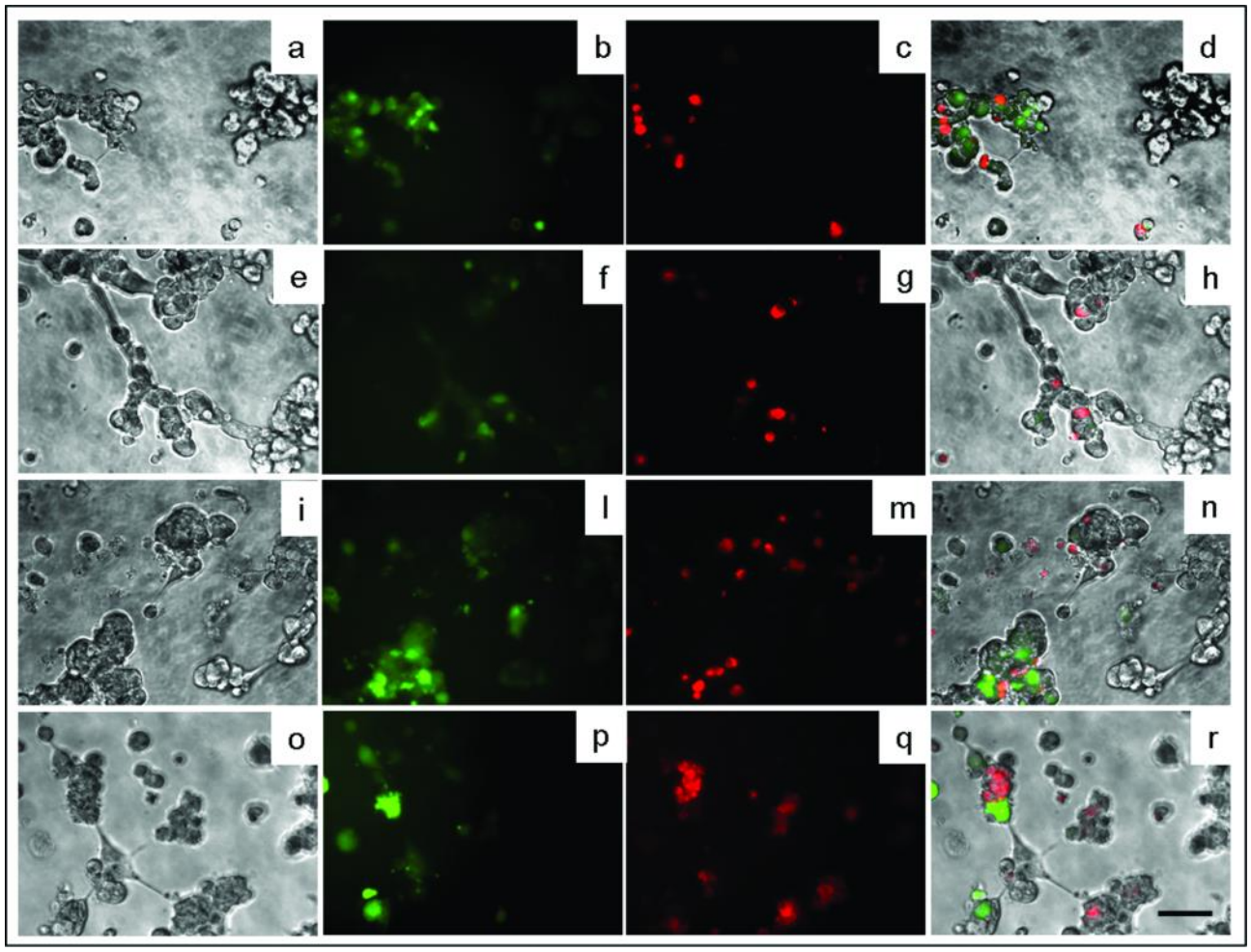

Figure 7

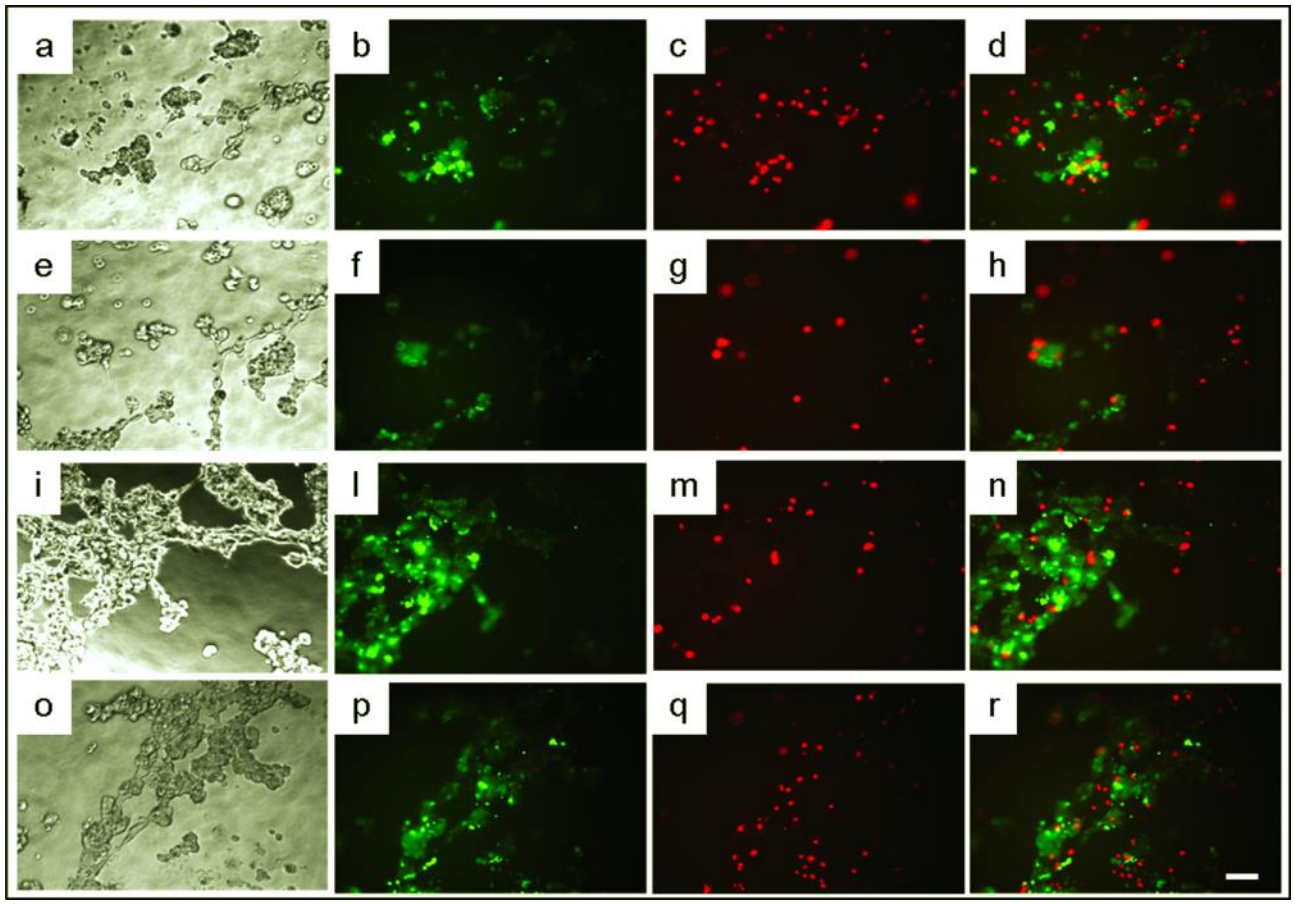

Figure 8