

Catalpol prevents glomerular angiogenesis induced by advanced glycation end products via inhibiting Galectin-3

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Research Article

Keywords: Catalpol, Glomerular angiogenesis, Advanced glycation end products, Galectin-3

Posted Date: March 21st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1399799/v1>

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Abstract

Objectives: The main characteristics of Diabetic Nephropathy (DN) at the early stage is abnormal angiogenesis of glomerular endothelial cells and macrophage infiltration. Galectin-3 plays a pivotal role in the pathogenesis of DN via binding with its ligand, Advanced Glycation End products (AGEs). Catalpol (Cat.), an iridoid glucoside extracted from *Rehmannia glutinosa*, has been found to ameliorate vascular inflammation, reduce endothelial permeability and protect endothelial damage upon diabetic milieu. However, little is known about whether Cat. could exert anti-angiogenesis and anti-inflammation effect induced by AGEs.

Methods: The anti-angiogenic mechanisms of Cat. were examined by incubating mouse glomerular endothelial cells (mGECs) and RAW 264.7 macrophages induced by AGEs. The expression of Galectin-3 and correlated molecules were also detected in diabetic rats treated with Cat.

Key findings: We observed that Galectin-3/Hypoxia-inducible factor-1 (HIF-1 α) was activated by AGEs in mGECs and RAW 264.7 macrophages, catalpol significantly inhibited the proliferation of the mGECs and RAW 264.7 macrophages *in vitro*. Meanwhile, it also ameliorated diabetes-induced renal damage via inhibiting Galectin-3/VEGF/HIF-1 α pathways *in vivo*.

Conclusions: Altogether, our findings demonstrated that Cat. prevent the angiogenesis of mGECs and macrophages proliferation via inhibiting Galectin-3. We suggest that Cat. contributes to block the pathogenic development of diabetes-induced renal damage.

Introduction

The pathogenesis of DN is observed as mesangial matrix expansion, glomerular inflammation ^[1], in the kidney sample obtained from Type 1 diabetic patients ^[2, 3], the increased density of microvascular injury caused by glomerular angiogenesis and increased efferent arterioles have been found. Angiogenesis, a new blood vessel sprouts from the existing vascular bed, which mediate the physiological process like embryonic development, wound healing and female menstruation. Nevertheless, it also occurs many pathological processes like tumor growth and metastasis, ischemia, arthritis and diabetic complications ^[4, 5, 6]. Endothelial matrix degradation, the migration, proliferation and tube formation of endothelial cells are involved in the angiogenic process. It has been acknowledgeable that immature vessel formation in the kidney aggravates the symptom of DN. Morphological studies have confirmed that abnormal new vessel is accompanied with renal edema, glomerular vessel injuries fibrinoid lesions, tubulointerstitial inflammation and urinary albumin excretion ^[3]. An effective way of protecting kidney of DN is blocking the glomerular abnormal angiogenesis ^[7].

Galectin-3 is a 32–35 kDa multi-functional protein, it widely expresses in tissue cells, e.g. endothelial cells, macrophages and etc. Galectin-3 mediates various biological functions, such as cells attachment, differentiation and proliferation, inflammatory infiltration, cancer invasion and angiogenesis ^[8, 9]. AGEs

are formed by their aldehyde functional group of the sugar with an amine or guanidine group of the amino acid via non-enzymatic glycation reactions, the increased accumulation of AGEs directly causes severe metabolic disorders. Being a receptor of AGEs, Galectin-3 participates in the pathogenesis of DN, some studies suggest that AGEs-Galectin-3 bindings show a protective role via reducing the uptake and tissue accumulation of these by-products. Furthermore, Galectin-3 independently plays a predominant role in modulating inflammation and fibrogenesis^[10]. Although controversies still exist, it indicates that galectin-3 mediates the progress of DN.

The vascular endothelial growth factors (VEGFs) are correlated with the function of kidney via mediating the process of angiogenesis^[11]. Galectin-3 participates in the VEGF-A induced-angiogenesis by modulating vascular endothelial growth factor-2 (VEGFR-2) phosphorylation and internalization^[12]. Ang-1 acts on the integrity of endothelial cells and promotes the formation of abnormal vessels via binding to its receptor Tie-2^[13]. Meanwhile, the natural antagonist of Ang-1, Ang-2, promotes VEGF-induced angiogenesis by loosening the attachment of endothelial cells^[14]. Moreover, HIF-1 α is a key protein mediating the fibrogenesis in DN models^[15]. Hypoxia results in the production of VEGF for angiogenesis^[16]. AGEs induce oxidative stress via up-regulating the expression of HIF-1 α . Altogether, it suggests that Ang-1, Ang-2, Tie-2, HIF-1 α are indispensable factors that promote angiogenic process. Although the mechanism of angiogenesis is complicated. Researchers have developed several compounds and monoclonal antibodies to block the angiogenic process^[17], anti-angiogenic therapy will become an attractive strategy to block DN.

Cat. is a famous molecule of iridoids with broad range of bio-activities. Recently, it has been demonstrated to enhance immune therapy in treatment of asthmatic mice^[18]. Cat. was reported to inhibit TGF- β 1-induced epithelial-mesenchymal transition in human non-small-cell lung cancer cells^[19]. In addition, Cat. suppresses CT26 colon cancer by inhibiting angiogenic markers VEGF, VEGFR2, HIF-1 α ^[20]. Furthermore, anti-cardiomyocyte apoptosis effect of catalpol in diabetic cardiomyopathy have also been confirmed yet^[21].

We previously reported that 50 μ g/mL AGEs could induce angiogenesis of mGECs in vitro, Cat. ameliorated glomerular dysfunction upon the stimulation of 200 μ g/mL AGEs^[22]. Since clearing the waste or metabolic by-products through the transportation of vasculature in our human body, the relationship between AGEs/Galectin-3-mediated angiogenesis and the effect of Cat. is still unknown. In the present study, we test the hypothesis that Cat. inhibits the angiogenesis of mGECs and proliferation of macrophages induced by 50 μ g/mL AGEs via blocking the expression of Galectin-3 and correlated pro-angiogenic factors.

Materials And Methods

Drugs and reagents

Cat. was purchased from Chengdu Herbpurify Co. Ltd. (Chengdu, China), the purity of Cat. was $\geq 98\%$ (Batch No. Z-005131123). High-glucose Dulbecco's Modified Eagle Medium (DMEM) and RPMI1640 were from GE Lifesciences (Hyclone, Logan, UT, United States). Fetal bovine serum (FBS) was purchased from Gemini Bio-products (Woodland, CA, United States). Radio Immunoprecipitation Assay (RIPA) lysis buffer, 5×Protein Loading buffer, Acrylamide/Methylene Bisacrylamide(29:1) Solution, 1.5M Tris(pH = 8.8), 1M Tris(pH = 6.8), N,N,N',N'-Tetramethylethylenediamine(TEMED), 100×penicillin–streptomycin, blocked goat serum, 4',6-diamidino-2-phenylindole(DAPI) were from Solarbio Company Ltd. (Beijing, China). Whole protein extraction kit was purchased from Keygene Biotech Corp.Ltd. (Nanjing,China). VEGFR-2, HIF-1 α , Goat anti-rabbit IgG Horse Radish Peroxidase (HRP) conjugated, goat anti-mouse IgG HRP conjugated, goat anti-rabbit fluorescein isothiocyanate (FITC), and goat anti-rabbit Cy3 were purchased from Boster Biological Technology Ltd. (Wuhan, China). Ponceau S stain solution, cell counting kit-8 (CCK-8), and lactate dehydrogenase (LDH) release assay kit were from Beyotime Biotechnology (Shanghai, China). VEGFR-1 and Galectin-3 antibodies were purchased from Proteintech Group, Inc. (Rosemont, IL). β -actin were purchased from Bioworld Technology, Inc. (Nanjing,China). Bovine serum albumin (BSA) was purchased from Biofroxx (Einhausen Deutschland). Galectin-3, VEGFR-A, Ang-1 and Ang-2 enzyme-linked immunosorbent assay (ELISA) kit were purchased from Shanghai Mlbio(Shanghai, China). PageRuler™ Prestained Protein Ladder, 10 to 180 kDa were purchased from ThermoFisher Scientific (Grand Island, NY)

Preparation of AGEs

The preparation of AGEs were described according to the previous literature ^[22]. Briefly, 5g/L BSA and 50mM D-glucose was dissolved in the 10 mM Phospho-Buffer Solution (PBS) (pH = 7.4), then the mixture liquid was incubated in a cell incubator (Sanyo, Osaka, Japan) for 3 months. Finally, AGEs were harvested by dialyzing the newly-formed brown mixture for 12h.

Culture of mGECs and RAW 264.7 cells and treatment

mGECs and RAW 264.7 cells were purchased from BeNa culture collection (Beijing China). In a 95% air/5% CO₂ incubator, mGECs were grown in the medium with high-glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. RAW 264.7 cells were cultured by RPMI1640 supplemented with 10% FBS and 1% penicillin-streptomycin. 80%-90% confluence of the cells were replaced by serum free cultural medium. Cell proliferation was arrested under this condition for 18-24h. Cat., GB1107 and adenovirus were administered 30 min prior to the stimulation of AGEs and incubated for 48h.

Animal experiments

The experimental protocols using animals were approved by the Institutional Animal Care and Use Committee of Nanjing university of Chinese medicine (approval no.ACU-23(20161228), Date of Ethical Approval: December 28,2016). Adult male wistar (~ 220-240g) rats were adaptively fed for five days in an air-conditioned environment(20 $\pm$ 2°C) with 12-hour light/dark cycles. In this study, the animal model method was as described as previously ^[23], in general, A DM model of male rats was induced with streptozotocin injection (30 mg/kg, i.p.) and high-fat diet. rats were divided groups at random. Apart from

blank control, 100mg/kg Cat.and 10mg/kg GB1107 was intragastric administered for 12 weeks, animals were sacrificed and renal cortices were harvested.

Adenovirus preparation and transfection.

We amplified the coding sequence of Galectin-3 by RT-PCR and ligated them into the GV-314 plasmid (Shanghai GeneChem) to produce Ad-Galectin-3-GFP. As a control, we also generated an adenoviral vector that expresses GFP alone (LV-GFP). We injected Ad-Galectin-3-GFP or Ad-GFP into the tail vein of the experimental animals for *in vivo* study. 48 hours after transfection, mGECs and renal cortices were harvested for further experiments respectively.

Western blotting

The procedure of western blotting was performed as described previously [22], cells were rinsed with ice-cold PBS. Lysates were harvested by ice-cold RIPA lysis buffer and centrifuged at 12,000 round per minute (RPM) for 10 min. Proteins were separated by 10% dodecyl sulfate sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto Polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The membranes were blocked with 5% BSA, the blots were incubated with Galectin-3 (1:1000), HIF-1 α (1:1000), VEGFR-1 (1:1000), β -actin (1:1000) at 4°C overnight. On the next day, membranes were washed by Phosphate Buffered Saline Tween-20 (PBST) and incubated with HRP bound secondary antibodies (1:5000) at room temperature. Finally, enhanced chemiluminescence kits (Thermo Scientific, IL) were used for detecting the immunoblotting bands. The quantification of the proteins was performed in the ImageLab 4.0 software (2011 Bio-Rad Laboratories).

Immunofluorescence

After 1×10^4 cells /well mGECs and macrophages being treated for 48 h, cells were rinsed with PBS (pH 7.4) and fixed with fresh 4% paraformaldehyde for 15 min. Fixed cells were blocked with 3% normal goat serum, 0.1% (w/v) Triton X-100, 0.3% BSA at room temperature for 1 h, and then incubated with primary antibodies at 4°C overnight. Galectin-3 (1:200), HIF-1 α (1:100), VEGFR-1 (1:100) and VEGFR-2 (1:200) were subsequently incubated overnight, cells were rinsed with PBS and incubated with goat anti-rabbit Cy3 (1:100) and goat anti-mouse FITC (1:100) at room temperature for 2 h. Finally, cells were stained with DAPI to label the nuclei, photographed using a fluorescence microscope (Nikon Ti-U, Japan), and analyzed with the image J. software.

Cell viability assay

Cell viability was measured by a CCK-8 kit. Cells were harvested and seeded at a concentration of 2×10^3 cells/well in 96 microplates, after 24 h incubation, cells were treated with 10 μ M Cat, GB1107 and adenovirus, 48 h later, 50 μ g/mL AGEs were added into each well. At the end of each time point, 10 μ L 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium was reduced by dehydrogenases in cells yielding an orange colored product (formazan). Absorbance was read using a microplate reader at 450 nm (Biotek Synergy HT).

Measurement of Ang-1, Ang-2 and Tie-2

mGECs were seeded in a 24-well plate at 4×10^5 cells/well. The cells were coincubated with 10 μ M Cat, GB1107 and adenovirus, 48h later, 50 μ g/mL AGEs were added in the medium, BSA was used as a negative control. Cell lysates were harvested by using ice-cold RIPA lysis buffer and centrifuged at 12,000 rpm for 10 min. The Ang-1, Ang-2 and Tie-2 detected assays were carried out using ELISA kits (Mlbio, Shanghai, China).

Statistical analysis

Data were expressed as mean $\pm$ SEM, and $p < 0.05$ was considered as significantly different. Comparison among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by bonferroni post hoc test.

Results

Cat. blocked the proliferation of mGECs and macrophages induced by AGEs.

We have previously mentioned that 50 μ g/mL AGEs exhibits pro-angiogenesis, but little is known about its potential mechanisms. Galectin-3 modulates angiogenesis [8] and inflammation [24], which is also considered as a receptor of AGEs. Therefore, we attempted to explore whether the secreted level of Galectin-3 and VEGF were affected by the concentration of AGEs in vitro. Consistent with the previous study, we found that the secreted Galectin-3 level from 50 μ g/mL AGEs –stimulated mGECs was significantly higher than that from 0 μ g/mL AGEs–stimulated mGECs for 48h (Fig. 1A). Meanwhile, similar result was observed in the secreted VEGFA-detected groups (Fig. 1B). It indicated that the level of Galectin-3 and VEGFA released from mGECs are closely related to the proliferation of mGECs. To test whether the level of Galectin-3 and VEGFA [25] secreted from macrophages are affected by the concentration of AGEs, macrophages were incubated with 0, 50, 100, 200, 400 μ g/mL AGEs, and the level of Galectin-3 and VEGFA was detected in each group at the time point of 0, 6, 12, 24 h. The results also showed that Galectin-3 and VEGFA secreted from macrophages reached the highest level when incubated with 50 μ g/mL AGEs for 48h (Fig. 1C and D). Nevertheless, the increased expression of Galectin-3 and VEGFA in mGECs and macrophages were gradually reversed when stimulated by 100, 200 and 400 μ g/mL AGEs (Fig. 1A, B, C and D). Then, we administered 10 μ M Cat. into the mGECs prior to the stimulation of 50 μ g/mL AGEs. After 48h incubation, the results showed that 10 μ M Cat. significantly blocked the proliferative status of mGECs and macrophages stimulated by 50 μ g/mL AGEs, the Galectin-3 inhibitors GB1107 also showed similar results. Meanwhile, overexpressed Galectin-3 abrogated the inhibitive effect of Cat. These data suggest that the content of Galectin-3 and VEGFA released from mGECs and macrophages is upregulated when induced by 50 μ g/mL AGEs, furthermore, Cat. inhibits the proliferation of mGECs and macrophages upon the stimulation of 50 μ g/mL AGEs via inhibiting Galectin-3.

Cat. decreased the level of Ang-1, Ang-2 and Tie-2 secreted from mGECs induced by AGEs

Angiogenic factors mainly operate the angiogenesis processes via regulating complicated signal transduction events. Ang-1, Ang-2, and their receptor tyrosine kinase-2 receptor (Tie-2), which are mainly expressed in endothelial cells^[26], have been identified to play a dominant role in the formation of blood vessels and angiogenesis upon hypoxia^[27]. These factors contribute to the stability of endothelial-endothelial and endothelial-peri endothelial cell interactions^[28]. To evaluate the released changes of these pro-angiogenic factors induced by 50µg/mL AGEs and the efficacy of Cat. The mGECs were incubated with Cat. 30 min prior to the stimulation of 50µg/mL AGEs. After incubation for 48h, ELISA kits were used to detect the released level of Ang-1, Ang-2, and Tie-2. We found that 50µg/mL AGEs strongly promoted the release of Ang-1, Ang-2 and Tie-2 from mGECs. Cat. significantly reversed the high release of these pro-angiogenic factors from mGECs induced by AGEs in vitro (Fig. 2A, B, C). Overexpression of Galectin-3 abrogated the inhibitive pro-angiogenic factors of Cat from mGECs. These results show that Cat. can inhibit the high expression of pro-angiogenic factors induced by 50µg/mL AGEs via inhibiting Galectin-3.

Cat. inhibited the expression of Galectin-3/VEGF/HIF-1α in mGECs induced by AGEs

Galectin-3 is not only secreted from tumor cells, but also it is highly expressed in vascular endothelial cells. The function of activated Galectin-3 is to induce angiogenesis by the demonstration that it induced 3D morphogenesis of endothelial cells and an angiogenic response in vivo^[29]. To further determine the Galectin-3/VEGF/HIF-1α pathway involves in the anti-AGEs-induced angiogenic effect of Cat. We detected the expression of Galectin-3, HIF-1α, VEGFR1 and VEGFR2 in the whole extraction of cytoplasm and membrane in the mGECs, the immunofluorescence results showed that 50µg/mL AGEs increased these proangiogenic factors in the whole cells, especially in the cytoplasm, Cat. inhibited the high expression of Galectin-3, HIF-1α, VEGFR1 and VEGFR2 (Fig. 2A, B, C and D). Furthermore, we measured the level of these factors by Western blotting. Consistent with the IF results, Galectin-3/VEGF/HIF-1α pathway was activated by 50µg/mL AGEs. Cat. could inhibit this pathway induced by 50µg/mL AGEs for 48h (Fig. 2E, F, G and H). Thus, the AGEs-activated Galectin-3/VEGF/HIF-1α pathway is involved in the anti-angiogenic effect of Cat..

Cat. suppressed the expression of Galectin-3/HIF-1α in macrophages induced by AGEs

Galectin-3 is widely distributed and secreted from the macrophages^[30]. Moreover, galectin-3 can aggravate the inflammation via macrophage activation and vessel lesions^[31]. There is evidence suggesting that the density of macrophages is closely associated with the angiogenic activity in diabetic retinopathy^[32]. Recently, a report showed that the macrophage from kidney promote the angiogenic environment under ischemia conditions^[33]. To explore whether Galectin-3/HIF-1α pathway involves in the

anti-proliferative effect of Cat. on macrophages, we seeded RAW 264.7 macrophages in plates in vitro. After cocultured with Cat. and 50µg/mL AGEs for 48h, the pro-angiogenic factors HIF-1α and Galectin-3 in macrophages were detected by IF assay. The results showed that 50µg/mL AGEs increased the expression of HIF-1α and Galectin-3 in the cytoplasm of macrophages, Cat. apparently decreased the level of HIF-1α and Galectin-3 in macrophages (Fig. 3A and B). In addition, the expressed level of HIF-1α and Galectin-3 was measured by western blotting. The Galectin-3/ HIF-1α pathway was activated by 50µg/mL AGEs. Similar to the mechanism interfered by Cat. in mGECs. Cat. attenuated the activated Galectin-3/ HIF-1α pathway stimulated by 50µg/mL AGEs(Fig. 3C). These results show that Cat. also inhibits AGEs/Galectin-3/ HIF-1α in macrophages, thus ameliorates the pathogenic conditions of angiogenesis.

Cat. deactivated the Galectin-3/VEGF/HIF-1α pathway of renal cortices in Diabetic rats

According to the previous studies, in diabetic rats, AGEs rapidly clear from the blood and then distribute in the tissues, eg. liver, lung, and kidney ^[23]. To explore whether the expression of Galectin-3 is affected by infused AGEs and relationship between AGEs-Galectin-3 axis and Cat. in the renal cortices, we examined the expression of Galectin-3, HIF-1α, VEGFR-1 and VEGFR-2. We found that Galectin-3 and downstream proangiogenic receptors were significantly increased compared with control group (Fig. 5A).It indicates that AGEs can promote the proliferation of endothelial cells in renal cortices. Meanwhile, when Cat. was orally administered to the animals, the expression of Galectin-3, VEGFR-1, VEGFR-2, HIF-1α was significantly decreased after infusion of AGEs for 8 consecutive days (Fig. 5A). Moreover, High expression of Galectin-3, F4/80 and CD34 were observed in the renal glomerulus of diabetic rats, Cat. significantly inhibited the expression of Galectin-3, macrophage infiltration and angiogenesis

in the kidney of diabetic rats(Fig. 5B),overexpression of Galectin-3 abrogated the inhibitive effects of Cat.which is consistent with the studies mentioned above. These results suggest that Cat. can ameliorate AGEs-induced renal damage via inhibiting Galectin-3/VEGF/HIF-1α pathways *in vivo*.

Discussion

AGEs are accumulated in circulation system and tissues.They mediate the progress of diabetic vascular complications, there is evidence suggesting that the amount of accumulated AGEs in target tissues are positively correlated with the severity of the pathogenic organs.AGEs can partly activate the release of proinflammatory factors via RAGE-dependent mechanisms to trigger the cellular damage and dysfunction ^[34]. The endothelium is a key structure in the development of vascular pathology in patients with renal damage caused by hyperglycemia ^[35]. The abnormal additional vessels in the kidney diabetic patients displays a thin wall at the basement membrane and the glomerular endothelial cells are swollen, suggesting they are not take effect in the physiologic progress .The monocytes/macrophages are easy to

infiltrate the vasculature. AGEs induces the secretion of adhesion molecules and chemokines in endothelial cells. As a result, monocytes/macrophages adhere firmly onto the monolayers, large quantities of inflammatory cells invading into the tissues^[36]. AGEs could enhance the expression of Cyr61, which activated PI3K/Akt pathway and promoted the expression of VEGF expression in RF/6A cell^[37]. The phosphorylation of moesin exerted its role in AGEs-mediated vascular barrier dysfunction, endothelial cell proliferation, and angiogenesis^[38]. We previously found that low concentration (~ 50–150 µg/mL) AGEs stimulated the proliferation of mGECs, whereas high concentration (~ 200–400 µg/mL) induced the cell apoptosis or death^[22]. In this paper, we further detected that the content of Galectin-3, VEGFA are increased after the stimulation of low concentration AGEs in mGECs and RAW264.7 cells, furthermore, Cat. significantly inhibited the proliferation of these cells after the stimulation of 50 µg/mL AGEs in vitro. These results demonstrated that Cat. can regulate the growth status of mGECs and RAW264.7 cells according to the concentration of AGEs. In the future studies, much emphasis must be laid on the anti-angiogenic effect of Cat. in animal models. A proper AGEs-induced angiogenic models are needed for further study.

Cat., a water-soluble bioactive iridoid from *Rehmannia glutinosa*, exhibited multiple pharmacological activities. For instance, Cat. took its great protective effects against adriamycin-induced nephropathy by inducing SIRT1-mediated inhibition of TRPC6 expression and enhancing MRP2 expression^[39]. Cat. suppressed osteosarcoma cell proliferation through blocking epithelial-mesenchymal transition (EMT) and inducing apoptosis^[40]. Cat. could be a novel drug candidate against myocardial ischemia for cardiovascular diseases^[41]. Nevertheless, little is known about the effect of Cat. on the early stages of diabetic complications. Previously, we found that cat. ameliorated the apoptosis or death of mGECs induced by 200 µg/mL AGEs. In this paper, we mainly detect the effect of Cat. on the proliferated status of mGECs induced by 50 µg/mL AGEs, mimicking the early pathogenic stages of DN in vivo.

It is confirmed that abnormal angiogenesis involved the pathogenesis of DN^[17]. At present, although the classic treatments for DN include inhibiting hyperglycemic, hyperlipemia, hypertension, and blocking renin angiotensin-aldosterone system (RAAS), there are still large number of patients suffering from the end-stage renal disease (ERSD) resulting from diabetes. Anti-angiogenic therapeutic strategy has been regarded as a novel treatment approach for treatment of DN. Cat. exerted anti-angiogenic effect on rat corneal neovascularization^[42]. In this study, we demonstrated that Cat. inhibited the release of Ang-1, Ang-2 and Tie-2 secreted from mGECs stimulated by 50 µg/mL AGEs. These results indicate that Cat. could be a potential candidate compounds from herbs for the treatment of abnormal neovascularization-related diseases.

Previous studies reported that the expression of Galectin-3 was highly correlated with the development of tumor and inflammatory diseases, such as pancreatic cancer^[43], wound healing and fibrotic processes^[44, 45]. In this study, based on Galectin-3 viewed as a receptor of AGEs, we found that Cat. inhibited expression of Galectin-3, HIF-1 α and related molecules stimulated by 50 µg/mL AGEs in mGECs and macrophages. It indicated that Cat. might possess anti-inflammatory and anti-angiogenic

properties. Moreover, much attention has been focused on the interaction between Galectin-3 and scaffold proteins. The interaction of galectin-3 and MUC1 extracellular domain promoted the MUC1 cell polarization and increased MUC1- Epidermal Growth Factor Receptor (EGFR) association, thus led to EGFR dimerization and activation in epithelial cancer cells [46]. Cystinosin, a critical regulator of galectin-3, was involved in cystinosis. It was found that galectin-3 interacted with the pro-inflammatory cytokine Monocyte Chemoattractant Protein-1, which stimulated the recruitment of monocytes/macrophages, and proved to be significantly increased in the serum of cystinosin^{-/-} mice and also patients with cystinosis [47]. It provides a new role for cystinosin and galectin-3 interaction and leads to identify new molecular targets to delay cystinosis progression. Moreover, Galectin-3 interacted with Domain 5 on CD146 eFL, because it contained poly-N-acetyllactosamine sites, and deletion of this domain significantly reduces binding, thus mediates endothelial cell migration [48]. Galectin-3 and galectin-3 binding proteins (G3BP) mediated the process of metastasis. Researchers found that treatment of dietary pectic polysaccharides significantly disrupted the interaction between galectin-3 and G3BP [49]. Rpph1 (ribonuclease P RNA component H1) directly interacted with Galectin-3 and up-regulated Galectin-3 expression and activity, in addition, Gal-3/Mek/Erk pathway was involved in the release of pro-inflammatory cytokines and proliferation of mesangial cells induced by Rpph1, which provided an approach for treating inflammation and proliferation of mesangial cells in DN [50].

Conclusion

We found the anti-angiogenesis effect of Cat. via inhibiting Galectin-3 (Fig. 6). The results emphasize that Galectin-3 plays a critical role in the abnormal angiogenesis and macrophage infiltration in the process of AGEs accumulation. As cerebral endothelial integrity [51] and nitric oxide [52] plays an indispensable role in maintaining the function of central nervous system, further studies are needed to explore whether Cat. could exert cerebrovascular or neuro-protective effect. Moreover, we demonstrated that Cat. ameliorated the proliferated status of mGECs and macrophages induced by AGEs via inhibiting Galectin-3. In addition, Cat. also decreased the high expression of VEGF and HIF-1 α induced by AGEs. The expression of Galectin-3 upon the accumulation of AGEs regulated by Cat. provides a potential therapeutic approach for tackling the abnormal angiogenesis at the early stage of DN.

Declarations

Author contributions

W.-X.S. and H.-Q.X. designed the research; W.-X.S., Y.C., Y.-Y.G., G.-H.L., and J.-F.L. performed the research; W.-X.S., and Y.C. analyzed the data; W.-X.S. and H.-Q.X. wrote the paper.

Conflict of Interest

This manuscript has not been published in whole or in part and is not being considered for publication elsewhere. This manuscript does not violate or infringe upon any existing copyright/s or license/s from

any third party. All authors contributed significantly to the work and are in agreement with the content of the manuscript. There are no conflicts of interests.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Acknowledgements

The study was supported by research grants from the National Natural Science foundation of China (No. 81374029, No. 81073111, No.81874359), Natural Science Foundation of the Higher Education Institutions of Jiangsu Province (18KJD360002),

a ProjectFunded by Jiangsu Agri-animal Husbandry Vocational College(NSF2021CB04),and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) (No.JKLPSE201604).

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Figures

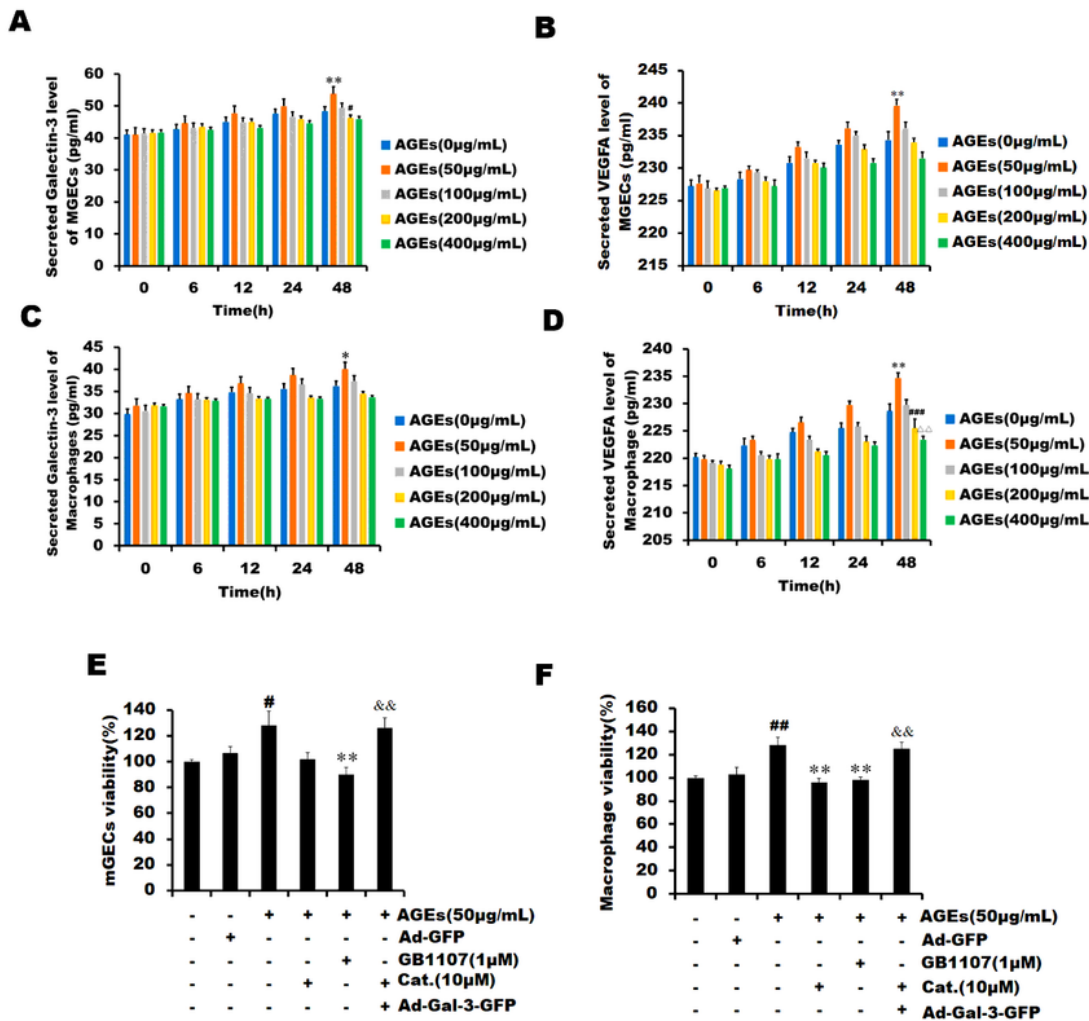


Figure 1

Cat. blocked the proliferation of mGECs and macrophages induced by AGEs.(A) Bar graph showing the secreted Galectin-3 level of mGECs when stimulated by AGEs at different concentrations (0, 50, 100, 150, 200, 400µg/mL) at 0,6,12,24and 48h using a Galectin-3 ELISA kit. ^{**} p<0.01compared with 50µg/mLAGEs at 0h, [#]p<0.05 compared with 200µg/mLAGEs at 0h group, n=3.(B) Bar graph showing the secreted VEGFA level of mGECs when stimulated by AGEs at different concentrations (0, 50, 100, 150, 200, 400µg/mL) at

0,6,12,24, and 48h using a VEGFA ELISA kit. ** $p < 0.01$ compared with 50 μ g/mL AGEs at 0h, n=3. (C) Bar graph showing the secreted Galectin-3 level of RAW264.7 cells when stimulated by AGEs at different concentrations (0, 50, 100, 150, 200, 400 μ g/mL) at 0, 6, 12, 24, and 48h using a Galectin-3 ELISA kit. * $p < 0.05$ compared with 50 μ g/mL AGEs at 0h, n=3. (D) Bar graph showing the secreted VEGFA level of RAW264.7 cells when stimulated by AGEs at different concentrations (0, 50, 100, 150, 200, 400 μ g/mL) at 0, 6, 12, 24, and 48h using a VEGFA ELISA kit. ** $p < 0.01$ compared with 50 μ g/mL AGEs at 0h, ### $p < 0.001$ compared with 200 μ g/mL AGEs at 0h group, $\Delta\Delta p < 0.01$ compared with 400 μ g/mL AGEs at 0h, n=3. (E) Bar graph showing the viability rate of mGECs when stimulated by 3×10^6 PFUAd-GFP, 50 μ g/mL AGEs, 50 μ g/mL AGEs+10 μ MCat., 50 μ g/mL AGEs+1 μ MGB1107, 50 μ g/mL AGEs+10 μ MCat.+ 1×10^6 PFUAd-Gal-3-GFP at 48h using a CCK-8 kit. # $p < 0.05$ compared with BSA group, * $p < 0.05$ compared with 50 μ g/mL AGEs group, n=3. (F) Bar graph showing the viability rate of RAW264.7 cells when stimulated by 3×10^6 PFUAd-GFP, 50 μ g/mL AGEs, 50 μ g/mL AGEs+10 μ MCat., 50 μ g/mL AGEs+1 μ MGB1107, 50 μ g/mL AGEs+10 μ MCat.+ 1×10^6 PFUAd-Gal-3-GFP at 48h using a CCK-8 kit. ## $p < 0.01$ compared with BSA group (negative control), ** $p < 0.01$ compared with 50 μ g/mL AGEs group, && $p < 0.01$ compared with 50 μ g/mL AGEs+10 μ MCat. group, n=3. All of the data were presented as means $\pm$ SEM and analyzed by one-way ANOVA followed by Bonferroni post hoc test.

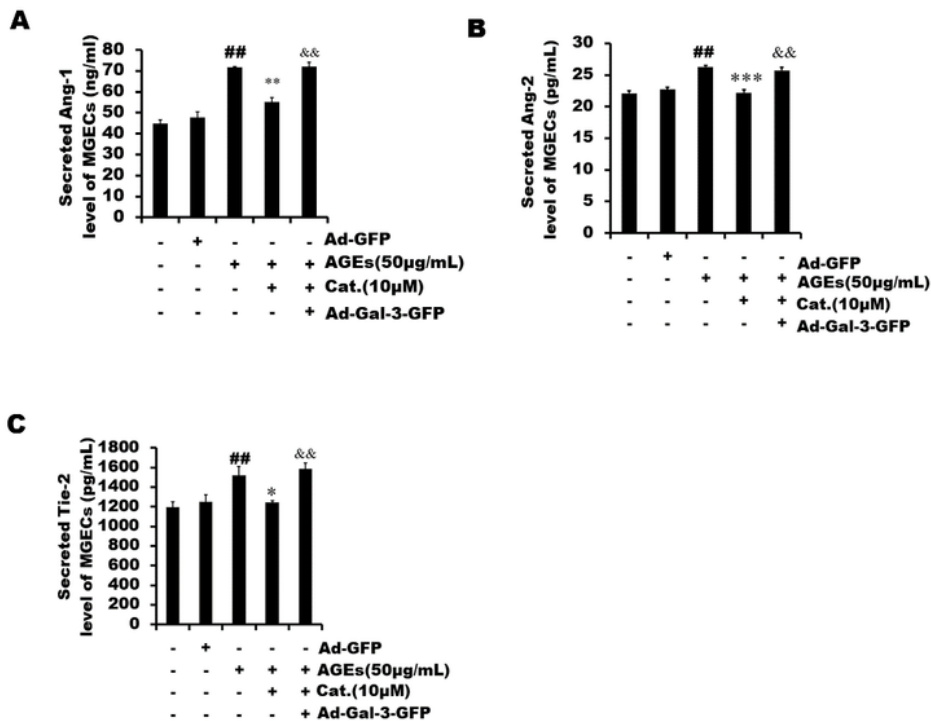


Figure 2

Cat. decreased the level of Ang-1, Ang-2 and Tie-2 secreted from mGECs induced by AGEs.(A) Bar graph showing the secreted Ang-1 level of mGECs when stimulated by 3×10^6 PFU Ad-GFP, 50µg/mL AGEs, 50µg/mL AGEs+10µM Cat., 50µg/mL AGEs+1µM MGB1107, 50µg/mL AGEs+10µM Cat.+ 1×10^6 PFU Ad-Gal-3-GFP at 48h using a Ang-1 ELISA kit. ## $p < 0.01$ compared with BSA group, * $p < 0.05$ compared with 50µg/mL AGEs group, $n = 6$. (B) Bar graph showing the secreted Ang-2 level

of mGECs when stimulated by 3×10^6 PFUAd-GFP, 50 μ g/mL AGEs, 50 μ g/mL AGEs + 10 μ M Cat., 50 μ g/mL AGEs + 1 μ M MGB1107, 50 μ g/mL AGEs + 10 μ M Cat. + 1×10^6 PFUAd-Gal-3-GFP at 48h using a Ang-2 ELISA kit. $^{##}p < 0.01$ compared with BSA group, $^{***}p < 0.001$ compared with 50 μ g/mL AGEs group, n=6. (C) Bar graph showing the secreted Tie-2 level of mGECs when stimulated by 3×10^6 PFUAd-GFP, 50 μ g/mL AGEs, 50 μ g/mL AGEs + 10 μ M Cat., 50 μ g/mL AGEs + 1 μ M MGB1107, 50 μ g/mL AGEs + 10 μ M Cat. + 1×10^6 PFUAd-Gal-3-GFP at 48h using a Ang-2 ELISA kit. $^{##}p < 0.01$ compared with BSA group, $^*p < 0.05$, $^{***}p < 0.001$ compared with 50 μ g/mL AGEs group, $^{&}p < 0.01$ compared with 50 μ g/mL AGEs + 10 μ M Cat. group, n=6. All of the data were presented as means $\pm$ SEM and analyzed by one-way ANOVA followed by Bonferroni post hoc test.

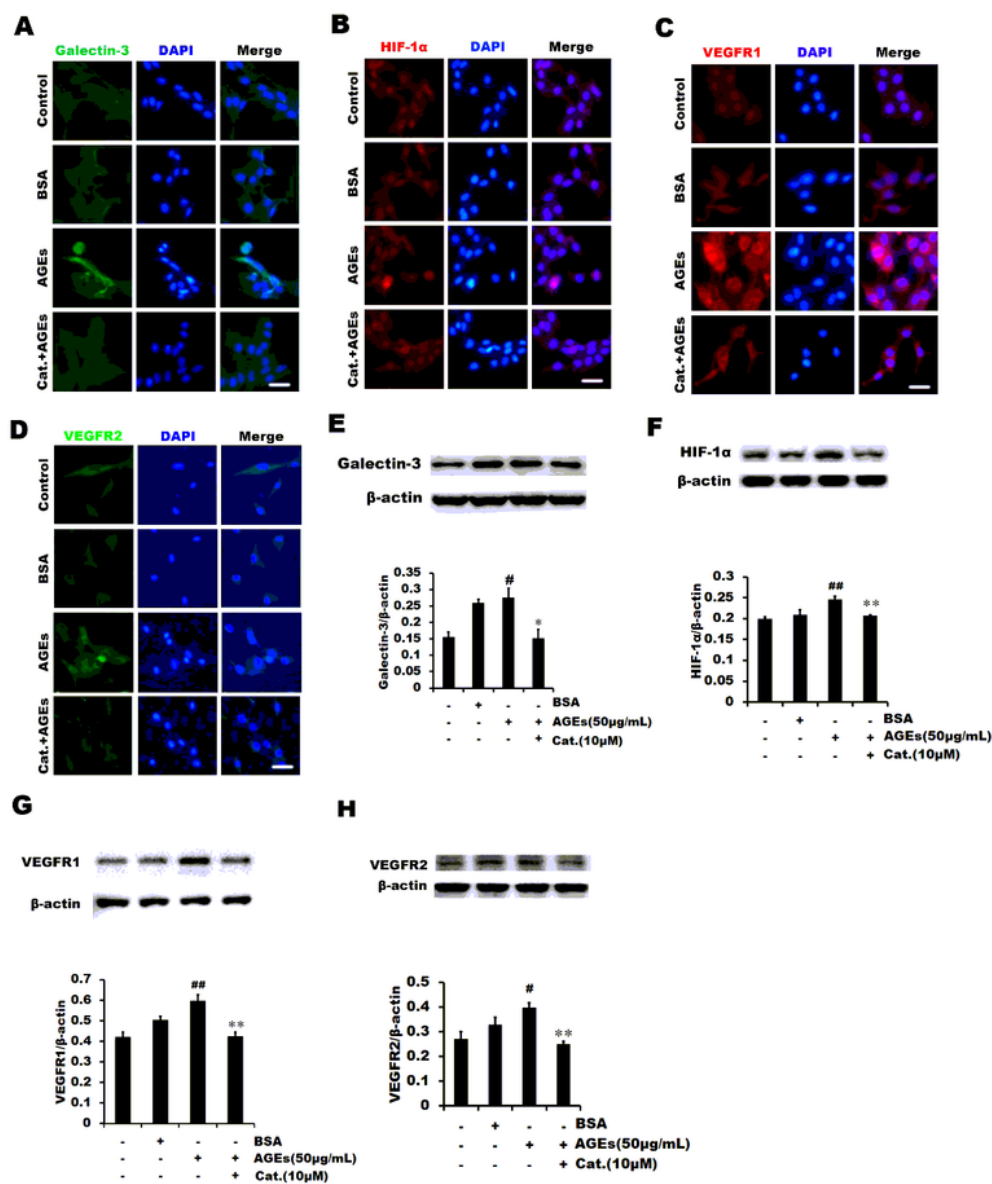


Figure 3

Cat. inhibited the expression of Galectin-3/VEGF/HIF-1α in mGECs induced by AGEs. (A) Representative immunofluorescence images of mGECs showing the Galectin-3⁺ cells when stimulated by 50μg/mL BSA, 50μg/mL AGEs and 50μg/mL AGEs+10μMCat. for 48h. (green: Galectin-3, blue: DAPI). (B) Representative immunofluorescence images of mGECs showing the HIF-1α⁺ cells when stimulated by 50μg/mL BSA, 50μg/mL AGEs and 50μg/mL AGEs+10μMCat. for 48h. (red: HIF-1α, blue: DAPI). (C)

Representative immunofluorescence images of mGECs showing the VEGFR1⁺ cells when stimulated by 50µg/mL BSA, 50µg/mL AGEs and 50µg/mL AGEs+10µM Cat. for 48h. (red: VEGFR1, blue: DAPI). (D) Representative immunofluorescence images of mGECs showing the VEGFR2⁺ cells when stimulated by 50µg/mL BSA, 50µg/mL AGEs and 50µg/mL AGEs+10µM Cat. for 48h. (green: VEGFR2, blue: DAPI). Representative western blotting and bar graphs to show the expression of (E) Galectin-3, (F) HIF-1α, (G) VEGFR1, (H) VEGFR2 of mGECs. #p<0.05, ##p<0.01 compared with BSA group, *p<0.05, **p<0.01 compared with 50µg/mL AGEs group, n=3, Scale bars, 200µm. All of the data were presented as means±SEM and analyzed by one-way ANOVA followed by Bonferroni post hoc test.

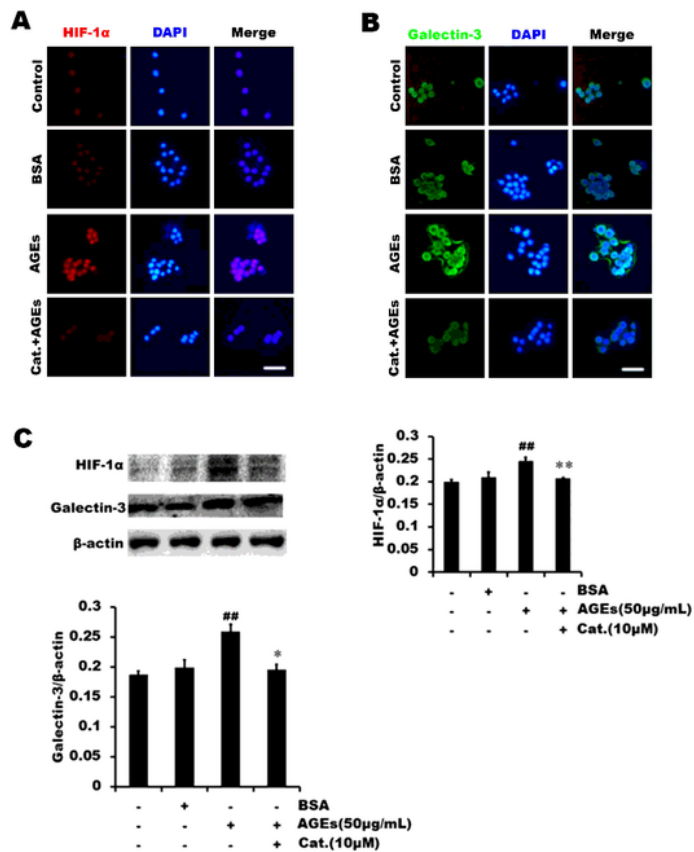


Figure 4

Cat. suppressed the expression of Galectin-3/HIF-1α in macrophages induced by AGEs.(A) Representative immunofluorescence images of RAW264.7 cells showing the HIF-1α⁺ cells when stimulated by 50μg/mL BSA, 50μg/mL AGEs and 50μg/mL AGEs+10μMCat. for 48h.(red:HIF-1α, blue: DAPI). (B)Representative immunofluorescence images of RAW264.7 cells showing the Galectin-3⁺ cells when stimulated by 50μg/mL BSA, 50μg/mL AGEs and 50μg/mL AGEs+10μMCat. for 48h.(green:Galectin-3, blue: DAPI).

(C) Representative western blottings and bar graphs to show the expression of Galectin-3 and HIF-1α of RAW264.7 cells ^{##}p<0.01 compared with BSA group, *p<0.05, **p<0.01 compared with 50μg/mL AGEs group, n=4, Scale bars, 200μm. All of the data were presented as means±SEM and analyzed by one-way ANOVA followed by Bonferroni post hoc test.

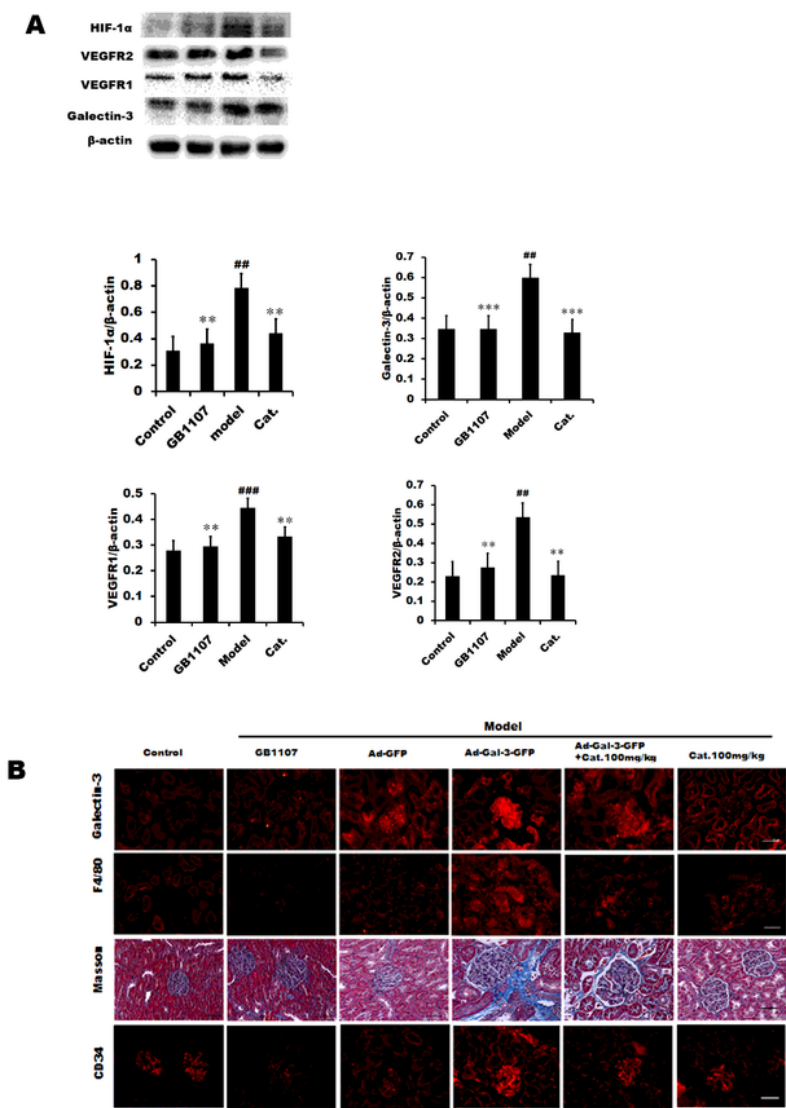


Figure 5

Cat. deactivated the Galectin-3/VEGF/HIF-1α pathway of renal cortices in diabetic rats.(A) Representative western blottings and bar graphs to show the expression of HIF-1α, VEGFR2, VEGFR1 and Galectin-3 of renal cortices.(B) Representative figures to show the expression of Galectin-3, F4/80(macrophage biomarkers),CD34(endothelium biomarkers), Masson staining to show the status of collagen accumulation in renal cortices [#]p<0.05, ^{##}p<0.01, ^{###}p<0.001 compared with BSA group, ^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001 compared with model group, n=12, Scale bars, 200μm. All of the data were presented as means±SEM and analyzed by one-way ANOVA followed by Bonfferoni post hoc test.

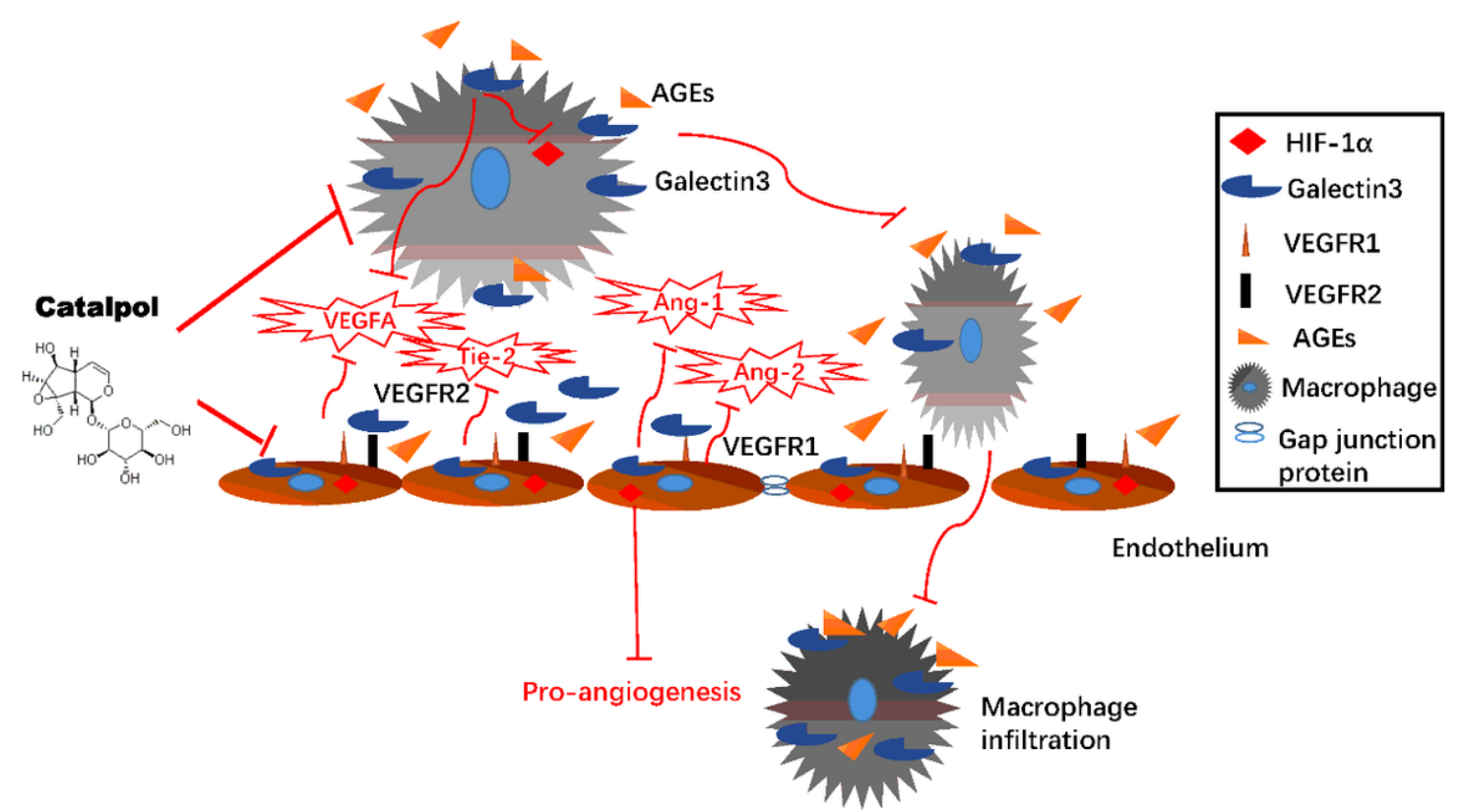


Figure 6

Cat. inhibited the angiogenesis of mGECs via inhibiting Galectin-3/VEGF/HIF-1α pathway upon 50μg/mL AGEs stimulation in vitro. Meanwhile, it suppressed the proliferation of macrophages via inhibition of Galectin-3 under the same condition. Cat. played a anti-angiogenic role in the early pathogenic changes of glomerular endothelium upon the induction of AGEs.