Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EXPRESSION AND LOCALISATION OF ADIPONECTIN AND ITS RECEPTORS IN HUMAN OCULAR TISSUES

VIDHYA SRINIVASAN[1#], PALANISAMY KARTHIKKA [1], KARUNAKARAN CORAL[1], SUBBULAKSHMI CHIDAMBARAM[1], PUKHRAJ RISHI[2] AND KONERIRAJAPURAM NATARAJAN SULOCHANA[1]*

[#]Ph.D Scholar, Birla institute of Technology and Science (BITS), Pilani, Rajasthan, India - 333 031
[1]R.S.Mehta Jain Department of Biochemistry and Cell Biology,
Vision Research Foundation, Sankara Nethralaya, Chennai, Tamil Nadu, India;
[2]Shri Bhagwan Mahavir Vitreo Retinal Services, Medical Research Foundation,
Sankara Nethralaya, Chennai, Tamil Nadu, India.

ABSTRACT

Recently we have reported the elevated levels of Adiponectin (APN) in proliferative diabetic retinopathy (PDR) vitreous in comparison to macular hole. It has been assumed that APN may get exudated into vitreous from circulation but no experimental proof has been shown so far. Therefore we set out to determine the origin of APN in the vitreous.Donor eye balls were used to extract RNA and protein for real time PCR, western blotting. Five micron retinal sections were done for immunofluorescence and immunohistochemistry. Primary retinal epithelial and endothelial cells were isolated from donor eye balls aged below 50 years.APN exerts its function through two receptors, AdipoR1 and AdipoR2. Interestingly we identified the expression of APN, AdipoR1 and AdipoR2 mRNA transcripts in all ocular tissues by quantitative PCR. Immunofluorescence and western blot analysis demonstrated the presence of APN and its receptors AdipoR1, AdipoR2 in all ocular tissues. AdipoR1 and AdipoR2 were distributed more in the retinal and choroidal layers as observed by immunohistochemistry. Surgically removed epiretinal membrane (ERM) showed a strong expression of mRNA transcript of APN and its receptors and immunofluorescence studies revealed the presence of protein as well. We have also confirmed the expression in primary human retinal endothelial cells (hREC), human choroidal endothelial cells (hCEC) and human retinal pigment epithelial cells (hRPE).Given the fact that APN regulates vasodilatation in retinal arterioles and inhibits choroidal neovascularization in animal models, it was unclear whether APN and it receptors exist in the human eye; our study has shown the localized expression of APN, AdipoR1 and AdipoR2 in different ocular tissues.

KEYWORDS: Adiponectin; AdipoR1; AdipoR2; hREC; hCEC; hRPE.

KONERIRAJAPURAM NATARAJAN SULOCHANA
R.S.Mehta Jain Department of Biochemistry and Cell Biology,
Vision Research Foundation, Sankara Nethralaya, Chennai, Tamil Nadu, India

INTRODUCTION

Adiponectin (APN) is a circulating peptide hormone mainly derived from adipocyte tissues. The oligomeric and globular forms of APN are regulators of energy homeostasis, which is reported to have anti-diabetic, anti-inflammatory, anti-angiogenic, anti-atherogenic and anti-hypertensive properties [1, 2]. AdipoR1 and AdipoR2 serve as receptors for globular and full-length APN. These receptors mediate AMP-activated protein kinases, the PPARα pathway, glucose uptake and the oxidation of fat in the liver and muscles [3]. APN suppresses VEGF-stimulated human coronary artery endothelial cell migration via cAMP/PKA-dependent signalling [4], which is an important effect that implies that APN is a regulator of vascular processes associated with diabetes and atherosclerosis. In addition to adipocytes, it is reported that APN is also synthesised and secreted in human and murine cardiomyocytes [5]. It is interesting to note that APN is expressed in choroidal tissues and is capable of inhibiting LASER-induced choroidal neovascularisation by up to 78% through intraperitoneal injections in an experimental mouse model [6]. It is reported that APN ameliorates retinopathy through the attenuation of inflammation, neovascularisation and fibrosis in mice [7], but to date, there is no clear report on whether APN, AdipoR1 and AdipoR2 are expressed in human ocular tissues. Few studies have reported the presence of APN in the subretinal fluid of patients with PVR, where it is significantly elevated and positively correlated with cathepsin. APN is also considered a predictor of redetachment in post-operative PVR [8].

Proliferative diabetic retinopathy (PDR) is a microvascular alteration that involves retinal endothelial dysfunction, vessel leakage, occlusion and retinal detachment. Elevated levels of aqueous humour APN in PDR patients were recently reported. These elevated levels may be a compensatory defensive mechanism that increases nitric oxide production, increases insulin sensitivity, and exhibits anti-inflammatory effects in PDR [9]. Earlier Zietz et al. reported a proof of concept regarding the presence of APN in the vitreous humour by measuring APN levels in only five human vitreous humour samples obtained after vitreoretinal surgery [10]. Zeitz et al. reported APN levels ranging from 2.0 - 70.2 ng/mL, which are in line with the findings of our study (2.28 to 301.8 ng/mL), in which a larger PDR sample size (*n*=29) was used [11]. The reported elevated levels of APN in the vitreous humour are too low when compared to blood, which ranges from 1 to 2 mg/dL. Still it is not know whether APN is synthesised intraocular or sequestered from the blood due to the breakdown of the blood-retinal barrier. To answer this question, we systematically examined human ocular tissues for APN, AdipoR1, AdipoR2 localisation and mRNA expression.

MATERIALS AND METHODS

Donor eyeball

All human studies were conducted in strict adherence to the guidelines of 'The Helsinki Declarations' and with the prior approval of the Institutional Review Board. Donor eyeballs were procured from the CU Shah Eye Bank (Sankara Nethralaya, Chennai) after the removal of the corneal buttons. Donor eyeballs were collected within 3-6 hours of death. The donor eyeballs collected were without any ocular disease and were procured with consent from the donors' relatives. Five donor eyeballs with a mean age of 65±7 years were used for the study.

Isolation of primary cell lines from donor eyeballs

The primary cell lines hRPE, hREC, and hCEC were isolated from the donor eyeballs. Donor eyeballs aged less than 50 years were used for the isolation of hRPE. The RPE explant was removed from the eyeballs carefully under aseptic conditions and gently placed on culture plates in accordance with the essential protocols of the isolation of hRPE as per Zhu et al. [12].

Donor eyeballs were collected in DMEM medium and processed on the same day. For the isolation of hREC, the retina was removed and minced into pieces. Cells were treated with 1.5% trypsin for 20 minutes at 37°C. The

trypsinised retina was homogenised and then aspirated through a syringe needle and further treated with 0.2 mg/mL collagenase for 40 minutes at 37°C. The collagenase treatment was terminated by adding medium containing 10 % FBS, followed by anti - CD31 magnetic bead separation at 4°C for 1 hour. Then, hREC was separated using a magnetic separator. hCEC isolation was also performed in the same way. All the cells were cultured in EGM (LONZA, USA) with 20 % FBS medium. Once the cells were grown, they were maintained in EGM with 2 % FBS. Endothelial cell specific marker Von Willebrand factor was used to confirm the primary cultures and the cells were used between 2 and 5 passages for the experiments.

Surgically removed ERM

Following vitrectomy, ERM was peeled to improve the visual acuity of the patient. Surgically removed ERMs were collected with the proper consent of the patient. The ERMs were transported from the operation theatre to the laboratory in normal saline and processed immediately.

Quantitative real-time PCR

RNA was extracted from the following donor eye tissues: iris, ciliary, choroid, retina, RBV, ONF, hRPE, hREC, hCEC and from ERM obtained from patients using TRI reagent. Approximately, 1 to 2 µg of RNA was reverse transcribed by an iScript cDNA synthesis kit (BioRad Laboratories, Inc., USA), and the resultant cDNA was used as a template for the amplification of APN, AdipoR1, and AdipoR2 [5]. Real-time PCR was performed using SYBR green PCR master mix (Eurogentec, Europe) on an ABI 7300 instrument. The values of specific genes were normalised to GAPDH.

Gene Name	Forward Primer	Reverse Primer
Human APN	5'- TGGTGAGAAGGGTGAGAA - 3'	5'- AGATCTTGGTAAAGCGAATC - 3'
ADIPOR1	5'- TTCTTCCTCATGGCTGTGATGT - 3'	5'- AAGAAGCGCTCAGGAATTCG- 3'
ADIPOR2	5'- ATAGGGCAGATAGGCTGGTTGA - 3'	5'- GGATCCGGGCAGCATACA- 3'
GAPDH	5'- GAACATCATCCCTGCCTCTACTG - 3'	5'- CGCCTCCTTCACCACCTTC -3'.

Quantitative PCR was carried out in triplicate.

Immunohistochemistry and immunofluorescence staining

Five-µm sections were made on paraffin blocks containing donor eyeballs and ERM obtained from PDR patients during intraocular surgery. Immunohistochemical staining for APN, AdipoR1 and AdipoR2 (Abbiotec, CA) was performed on these sections. After the deparaffinisation and rehydration of the sections, further analysis was carried out with a Novolink Min polymer detection kit (Leica biosystems, UK). The slides were then incubated with 1:200 diluted rabbit polyclonal antibody for APN/ AdipoR1 / AdipoR2, following the steps described by the supplier. Immunofluorescence staining was performed for APN, AdipoR1 AdipoR2 on tissue sections of donor eyeballs and ERM. In negative control specimens, the entire procedure was performed without primary antibody.

Quantification of APN in ocular tissues

Ocular tissues, including iris, ciliary, choroid, retina, ONF, and RPE, were isolated from the donor eyeballs. Protein was extracted using M-PER buffer (Pierce, USA). The protein concentrations of all ocular tissues and vitreous samples were estimated by the Bradford method (Pierce, USA). The total APN concentration in iris, ciliary, choroid, retina, ONF, and RPE isolated from donor eyeballs (*n*=3) were determined using a sandwich enzyme immunoassay according to the manufacturer's instructions (R&D, USA). The minimum detectable dose of the ELISA kit used was 0.24 ng/mL.

Electrophoresis and western blot

Seventy-five µg protein was mixed with 5× sample buffer (3 % SDS, 50 mmol/L TRIS HCl pH 6.8, 2 % mercaptoethanol, 10 % glycerol, 10 mmol/L DTT) [13] and boiled at 95°C for 10 minutes under fully denatured and reducing conditions and was then loaded onto 10 % SDS gel. Proteins separated were transferred to nitrocellulose membrane, and the membranes were blocked with 5 % milk PBST for one hour and incubated overnight at 4°C with 1:200 diluted primary antibodies (rabbit polyclonal antibodies, Abbiotec, CA).

The membranes were washed three times with PBST followed by anti-rabbit HRP (1:2500) incubation for 2 hours (Santa Cruz, USA). The membranes were washed three times, and chemiluminance was captured using a FluorChem FC3 (Protein simple, USA) with ECL (Pierce, USA) detection kit.

RESULTS

Intraocular expression of APN, AdipoR1 and AdipoR2

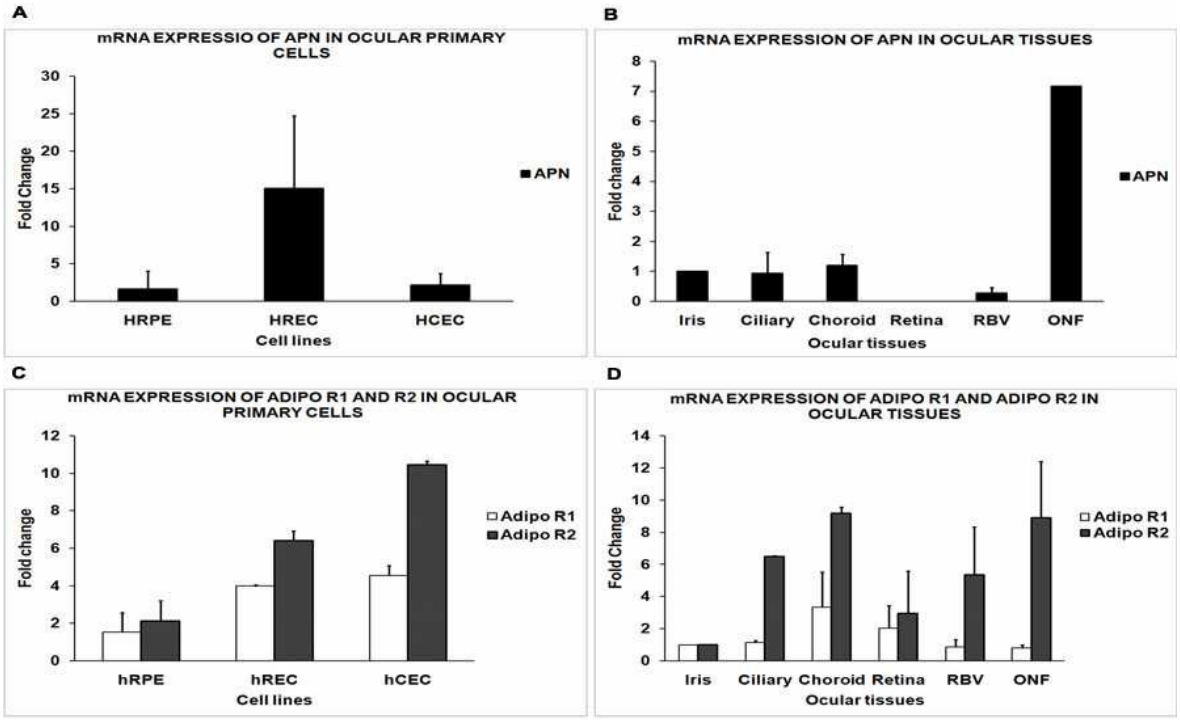
Real time PCR was done for APN, AdipoR1 and AdipoR2 in different ocular tissues and the values were normalised to endogenous control GAPDH. Expression of APN mRNA was almost equal in hRPE and hCEC whereas hREC showed very high levels (Fig.1A). AdipoR1 and adipoR2 transcripts were more in hCEC compared to hRPE and hREC. Expression of AdipoR2 was significantly more than AdipoR1 in all the three primary cultures (Fig.1C). Different ocular tissues, i.e., iris, ciliary, choroid, retina, retinal blood vessel and ONF were also analyzed for mRNA expression and the values were calibrated to iris to calculate the fold changes. Optic nerve fibre showed higher expression of APN followed by choroids, ciliary, iris and retinal blood vessels. The level of APN mRNA in retinal tissue was close to undetectable levels (Fig.1B). Both AdipoR1 and AdipoR2 were expressed in all ocular tissues but interestingly AdipoR2 expression was significantly higher (Fig.1C). Thus, we confirm the expression of APN and its receptors AdipoR1 and AdipoR2 in ocular tissues.

APN and its receptors are localised in human retinal tissues and epiretinal membrane

Immunohistochemistry revealed the presence of APN, AdipoR1 and AdipoR2 in different layers of human donor eye sections. Five donor eyeballs with no diabetic complication and a natural cause of death were used for staining. Figure 2 is a representative image showing the presence of APN, AdipoR1 and AdipoR2 in the human retina. The immunolocalisation of donor eyes showed weak signals of APN, whereas its receptors AdipoR1 and AdipoR2 showed strong signals in the retinal layers, RPE and choroid. We have also done immunofluorescence staining for APN, AdipoR1 and AdipoR2 to further confirm an immunohistochemistry observation (Fig.3). APN (Fig. 3A, D and G) and its receptors AdipoR1 (Fig. 3B, E and H) and AdipoR2 (Fig. 3C, F and I) are localised in the ciliary body, retina, choroid layers and also in ERM from PDR patients. The protein expression of APN in ocular tissue was measured quantitatively by ELISA where ONF showed maximum expression, followed by ciliary, choroid, retina, RPE and iris, successively (Fig.4A). Our western blot analysis confirmed the protein expression of APN, AdipoR1 and AdipoR2 (Fig.4B & 4C) in the ocular tissues.

Figure 1

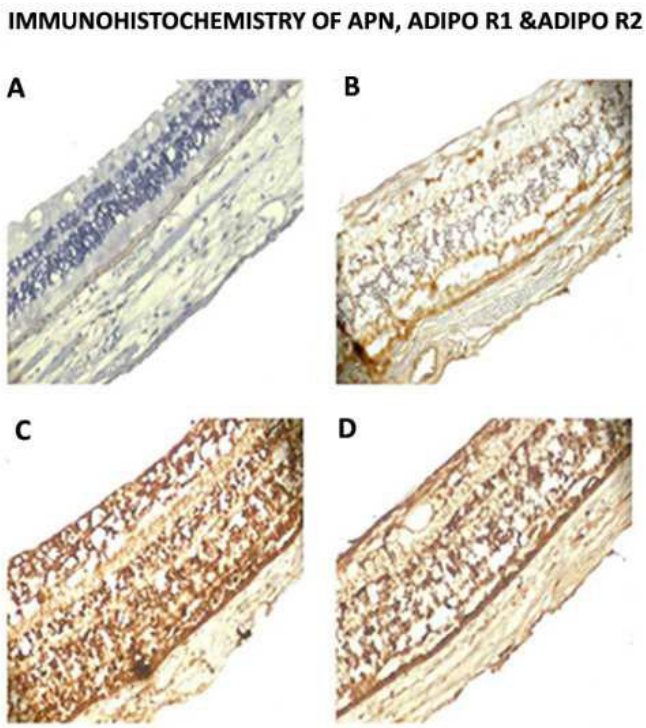
Intraocular mRNA expression studies for APN and its receptors by real-time PCR



a) Results of quantitative PCR for APN mRNA in ocular primary cells.
b) Results of quantitative PCR for APN in ocular tissues.
c) Results of quantitative PCR for AdipoR1 and AdipoR2 mRNA in ocular primary cells.
d) Results of quantitative PCR for AdipoR1 and AdipoR2 mRNA in ocular tissues.

Figure 2

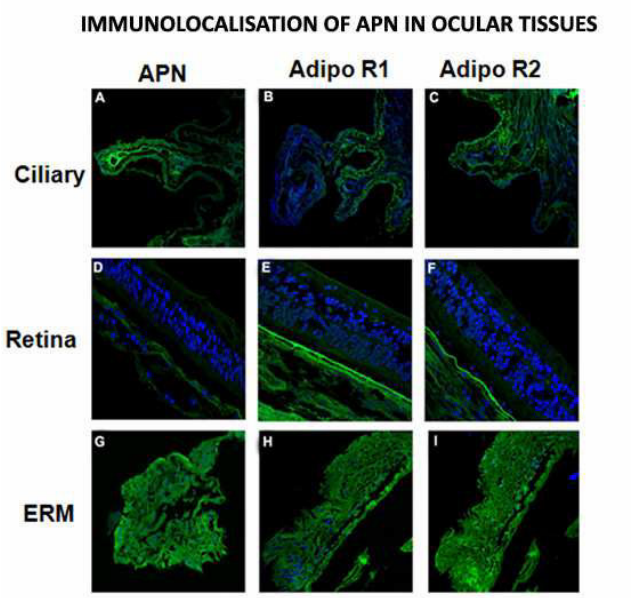
Immunohistochemistry of APN, AdipoR1 and AdipoR2 in ocular tissue



Representative immunohistochemical staining performed on 5-µm sections of paraffin block of a donor eyeball. A- Negative staining without primary antibody, B- APN, C- AdipoR1, D- AdipoR2.

Figure 3

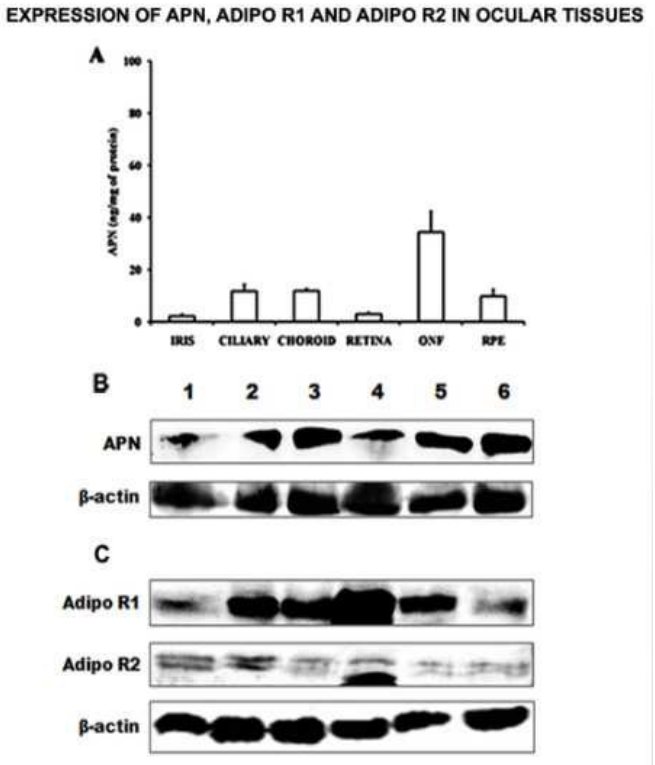
Confocal micrograph of APN, AdipoR1, AdipoR2 in ocular tissue and surgically removed membrane stained for APN (Fig: A,D,G), AdipoR1(Fig: B,E,H), AdipoR2(Fig: C,F,I)



A,B,C- Immunoflouresence staining of donor eyeball showing ciliary process; D,E,F- retinal and choroidal layers; G,H,I – surgically removed ERM.

Figure 4

Protein level expression of APN and its receptors in ocular tissues and vitreous



A. ***Quantification of APN protein expression by ELISA in ocular tissue.***
B. ***Western blot analysis of APN and beta actin in ocular tissue. Lane 1: iris, Lane 2: ciliary, Lane 3: choroid, Lane 4: retina, Lane 5: optic nerve fibre, Lane 6: RPE.***
C. ***Western blot analysis of AdipoR1, Adipo R2 and beta actin in ocular tissue. Lane 1: iris, Lane 2: ciliary, Lane 3: choroid, Lane 4: retina, Lane 5: optic nerve fibre, Lane 6: RPE.***

DISCUSSION

We recently reported elevated levels of APN in the vitreous humour of patients with PDR [11]. It is very intriguing how APN gains entry into the vitreous humour because adipocytes were once believed to be the only source of APN expression. It could be possible that, in patients with PDR, the retinal barrier is affected; thus, APN present in the blood could seep into the vitreous humour [9]. However, recent articles have demonstrated the presence of APN transcripts in myocytes [14], osteoblasts [15], liver endothelial cells [16] and placental tissue [17]. More interestingly, Bora et al. demonstrated the expression of APN in rat choroidal tissue and also reported that APN would be beneficial as an anti-angiogenic for treating laser choroidal neovascularisation [6]. The receptors AdipoR1 and AdipoR2 play a major role in mediating adiponectin signalling. It has been reported that brain endothelial cells also express both receptors but not APN [18]. Yoneda et al. reported the presence of AdipoR1 and AdipoR2 in normal colon epithelium as well as in colon cancer tissues and that APN plays a major role in the physiological and pathological condition of colon epithelium via its receptors [19]. In this study, we have reported the expression and localisation of APN and its receptors AdipoR1 and AdipoR2 in human ocular tissues. We report that APN and its receptors AdipoR1 and AdipoR2 mRNA are expressed in the retinal layers, iris, ciliary body, optic nerve fibre and choroidal layers and also protein expression, as detected by western blots. Though a previous study [11] reported elevated levels of APN in the vitreous humour of patients with PDR, as analysed by ELISA, here we confirm the same results by western blot analysis.

We also analysed the expression levels in primary cells, i.e., hREC, hCEC and hRPE, isolated from cadaver eyes. hREC cells showed higher expression of APN compared than hCEC and hRPE. In addition, surgically removed ERM showed higher expression of APN mRNA. Our results clearly demonstrate that APN and its receptors are endogenous, or locally expressed, throughout the human eye. The coexistence of APN and its receptors points to the possible APN signalling pathway in ocular tissues. Because the neural retina and hREC showed stronger expression of APN, we presume that elevated levels of APN in PDR vitreous humour could be produced by retinal microvascular endothelial cells and/or by the retinal ganglion layer. Although we confirm the intraocular expression of APN in this article, we still do not know the reason for the elevated levels in PDR. However, further studies on APN are needed to understand its role in normal ocular physiology and pathology. Abbreviations: APN – adiponectin, AdipoR1- adiponectin receptor 1, AdipoR2 – adiponectin receptor 2, PDR - proliferative diabetic retinopathy, MH – macular hole, PVR - proliferative vitreoretinopathy hRPE – human retinal pigment epithelial cells, hREC – human retinal endothelial cells, hCEC- human choroidal endothelial cells, VEGF – vascular endothelial growth factor, ERM – epiretinal membrane, ONF- optic nerve fibre, RBV- retinal blood vessels.

ACKNOWLEDGEMENT

The authors thank Vanitha, Harish Vuyyuru and Gomathy (Sankara Nethralaya, Chennai) for their technical assistance. We thank the Department of Biotechnology at IIT (Chennai) for confocal microscopy imaging. We acknowledge the project grant from ICMR 5/4/6/6/09 NCD II.

REFERENCES

1. Kawano J., Arora R., The role of Adiponectin in obesity, diabetes, and cardiovascular disease. J. Cardiometab. Syndr, 4: 44-49, (2009)
2. Shen YY., Peake PW., Charlesworth JA., Review article: Adiponectin: its role in kidney disease. Nephrology. (Carlton), 13:528-534, (2008)

3. Kadowaki T., Yamauchi T., Adiponectin and Adiponectin receptors. Endocr. Rev, 26 : 439-451, (2005)
4. Mahadev K., Wu X., Donnelly S., Ouedraogo R., Eckhart AD., Goldstein BJ., Adiponectin inhibits vascular endothelial growth factor-induced migration of human coronary artery endothelial cell. Cardiovasc. Res,78: 376-384 ,(2008)
5. Pineiro R., Iglesias MJ., Gallego R., Raghay G., Eiras S., Rubio J et al., Adiponectin is synthesized and secreted by human and murine cardiomyocytes. FEBS. Lett, 579: 5163-5169,(2005)
6. Bora PS., Kaliappan S., Lyzogubov W., Tytarenko .RG., Thotakura S., Vishwanathan T., Bora NS., Expression of Adiponectin in choroidal tissue and inhibition of laser induced choroidal neovascularization by Adiponectin. FEBS . Lett, 581: 1977-1982,(2007)
7. Krenning G., Moonen JR., Harmsen MC., Pleiotropism of Adiponectin: inflammation, neovascularization, and fibrosis. Circ. Res, 104:1029-1031,(2009)
8. Ricker Jag L., Kijlstra A., Kessels AGH., Jager Wd., Hendrikse F., Heij La EC., Adipokine levels in subretinal fluid from patients with rhegmatogenous retinal detachment. Exp. Eye. Res,94: 56-62,(2012)
9. Danna Mao., Hui Peng., Qiuhong Li., Jun Wang., Pinghua Li., Ke Hu1., Xuedong Zhang., and Bo Lei., Aqueous Humor and Plasma Adiponectin Levels in Proliferative Diabetic Retinopathy Patients. Current Eye Research,37: 803-08,(2012)
10. Zietz B., Buechler C., Kobuch K., Neumeier M., Scholmerich J., Schaffler A., Serum levels of Adiponectin are associated with diabetic retinopathy and with Adiponectin gene mutations in Caucasian patients with diabetes mellitus type 2. Exp. Clin. Endocrinol Diabetes,116: 532-36,(2008)
11. Vidhya S., Subbulakshmi C., Coral K., Pukhraj R., Sulochana KN., Measurement Of Adiponectin In Vitreous And Plasma Of Patients With Proliferative Diabetic Retinopathy And Its Correlation With Vascular Endothelial Growth Factor, Pigment Epithelial Derived Factor And Insulin Like Growth Factor-1. IJPBS, 4: 993-1005,(2013)
12. Zhu M., Provis JM., and Penfold PL., Isolation, culture and characteristics of human foetal and adult retinal pigment epithelium. Australian and New Zealand Journal of Ophthalmology, 26: S50-52,(1998)
13. Waki H., Yamauchi T., Kanon J., Ito Y., Uchida S., Kita S et al., Impaired multimerization of human Adiponectin mutants associated with diabetes, Molecular structure and multimer formation of Adiponectin. J. Biol. Chem,278: 40352-40363,(2003)
14. Aure´ lie m., jean-christophe j., isabelle., olivier c., and sonia m., Induction of Adiponectin in Skeletal Muscle by Inflammatory Cytokines: *in Vivo* and *in Vitro* Studies. Endocrinology,145: 5589–5597,(2004)
15. Berner HS., Lyngstadaas SP., Spahr A., Monjo M., Thommesen L., Drevon CA., Syversen U., Reseland JE., Adiponectin and its receptors are expressed in bone-forming cells. Bone,35: 842-9,(2004)
16. Kaser S., Moschen A., Cayon A., Kaser A., Crespo J., Pons-Romero F., Ebenbichler CF., Patsch JR., Tilg H., Adiponectin and its receptors in non-alcoholic steatohepatitis. Gut, 54 :117–121,(2005)
17. Eduardo CJ., Rube N., Rosalı G., Susana B., Sulay T.,Toma´s C., Felipe FC., and Carlos D., Expression and Regulation of Adiponectin and Receptor in Human and Rat Placenta. J. Clin. Endocrinol Metab, 90 :4276–4286, (2005)
18. Spranger J., Verma S., Gohring I., Bobbert T., J Seifert., Sindler AL., Pfeiffer A., Hileman SM., Tschop M and Banks WA., Adiponectin does not cross the blood-brain barrier but modifies cytokine expression of brain endothelial cells. Diabetes, 55: 141-7,(2006)
19. Yoneda K., Tomimoto A., Endo H., Iida H., Sugiyama M., Takahashi H et al., Expression of Adiponectin receptors, AdipoR1 and AdipoR2, in normal colon epithelium and colon cancer tissue. Oncology Reports, 20: 479-483,(2008)