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Esculetin attenuates alterations in Ang II and acetylcholine mediated vascular reactivity associated with hyperinsulinemia and hyperglycemia

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ABSTRACT

Esculetin (6, 7- dihydroxycoumarin) was found to be protective against hepatic and renal damage associated with Streptozotocin (STZ) induced type 1 diabetes, because of its radical scavenging property. However, there are no reports regarding its effect on vascular dysfunction under hyperinsulinemic and hyperglycemic conditions. Hence, the present study aimed to investigate the effect of esculetin on vascular dysfunction under these conditions. Non-genetic model of hyperinsulinemia and hyperglycemia were developed by high fat diet (HFD) feeding and HFD + Streptozotocin (STZ, 35 mg/kg, *I.P*) treatment in Wistar rats, respectively. Esculetin was administered at 50 and 100 mg/kg/day (*P.O,* 2 weeks) doses and biochemical, vascular reactivity and immunohistochemical experiments were performed to assess the effect of esculetin on vascular dysfunctions. Esculetin treatment significantly attenuates metabolic perturbations, alleviates insulin levels in hyperinsulinemic condition. Thoracic aorta of hyperinsulinemic and hyperglycemic rats showed hyper-responsiveness to Ang II mediated contraction and impaired acetylcholine mediated relaxation, and esculetin attenuates alterations in vascular reactivity to Ang II and acetylcholine challenges. In addition, immunohistochemical evaluations revealed that esculetin prevents increase in AT_1R, AT_2R, Keap1, TGF-β, and decrease in ACE2 expression in aorta of hyperinsulinemic and hyperglycemic rats.

1. Introduction

Among all complications associated with diabetes, vascular complications and thereby cardiovascular diseases (CVD) alone are the reason for more than 50% of diabetic patients' death [1]. Ang II, an integral component of renin angiotensin system (RAS) plays a pivotal role in the development of vascular complications. Ang II modulates a variety of physiological effects through Angiotensin II type 1 receptors (AT_1R) and Angiotensin II type 2 receptors (AT_2R) in numerous tissues including the vascular system [2]. Experimental and epidemiological studies suggest that hyperinsulinemia and hyperglycemia lead to activation of the RAS and up-regulation of AT_1R, which are considered to be the main culprit for the development of vascular complications associated with diabetes [2–4].

In the present study, we have used high fat diet (HFD) fed and HFD fed/Streptozotocin (STZ, 35 mg/kg, *I.P*) treated rats as non-genetic model of hyperinsulinemia and hyperglycemia which can mimic the altered metabolic events as seen in human type 2 diabetes [5]. HFD and STZ treated rats showed metabolic perturbations, hypertension, oxidative stress, over activation of renin-angiotensin system (RAS), and increased Ang II vascular reactivity which lead to vascular dysfunction [5]. Therefore, there is a need of novel therapeutic intervention which can prevent, reverse or delay these multiple alterations to address vascular dysfunction associated with hyperinsulinemia and hyperglycemia.

Esculetin a naturally occurring 6,7-dihydroxy derivative of coumarin was found to be protective against hepatic and renal dysfunction associated with diabetes in STZ induced type 1 diabetic rats [6–8]. Since ancient time esculetin containing plants are

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commonly used as a folk medicine to counteract various inflammatory, allergic and infectious diseases. Esculetin has been reported for inhibiting lipoxygenase and cycloxygenase [9], scavenging reactive oxygen species (ROS) [8], anti-fibrotic [7], and anti-proliferative [10,11] effects. However, effect of esculetin on diabetic vascular dysfunction is yet to be studied. A selective 5-lipoxygenase inhibitor (caffeic acid phenethyl ester) have been reported to reduce vascular hyper-responsiveness and vascular stiffness, thereby, alleviates diabetic atherosclerotic manifestations [12]. Further, metformin has been reported to restore endothelial function through inhibiting endoplasmic reticulum stress, oxidative stress and increasing NO bioavailability by activation of AMPK/PPARδ pathway in obese diabetic mice [13]. A recent report suggests that esculetin has anti-adipogenic effects through modulation of PPARγ and C/EBPα via the AMPK signaling pathway [14]. Based upon these findings, we hypothesized that due to ROS scavenging, lipoxygenase inhibitory potential and AMPK modulating effect, esculetin may attenuate Ang II mediated vascular hyper-responsiveness, alleviate impaired acetylcholine induced relaxation and prevent vascular dysfunction associated with type 2 diabetes.

2. Methods

2.1. Animal studies

The male Wistar rats (160–180 g) were procured from the central animal facility of the institute, BITS Pilani, and all the animal experiments were performed in accordance with the CPCSEA guidelines (Government of India). Animals were maintained under standard environmental conditions and provided with feed and water *ad libitum*. All the animals were fed on a normal pellet diet (NPD) one week prior to the experimentation. Hyperinsulinemia and hyperglycemia were induced by High Fat Diet (HFD) and HFD/STZ (35 mg/kg, *I.P*) treatment respectively, as per the protocol described by Gaikwad et al., [15]. Briefly, rats were allocated to two dietary regimens either Normal Pellet Diet (NPD, n = 24) or HFD (n = 36) *ad libitum* respectively for an initial period of 2 weeks. After 2 weeks of dietary manipulation, the rats from the HFD-fed group were injected with a low dose of STZ (35 mg/kg, *I.P*) (n = 18), whereas the respective control rats were given vehicle citrate buffer (pH 4.4). After 4 weeks, rats fed with NPD, HFD and HFD + STZ were treated with esculetin (Sigma Aldrich) 50 and 100 mg/kg/day, *P.O* and respective control animals treated with vehicle (0.5% sodium carboxy methyl cellulose) for 2 weeks and the rats were allowed to feed on their respective diet till end of the study (n = 6) [7]. Body weight, biochemical estimations, and blood pressure measurements were performed at the end of 6 weeks.

2.2. Assessment of biochemical and hemodynamic changes

The blood samples were collected, plasma was separated and fasting plasma glucose (PGL), triglycerides (PTGs), and total cholesterol (PTC) were estimated as per manufacturer's instructions by using commercially available spectrophotometric kits (Accurex Biomedical Pvt. Ltd., Mumbai, India). Plasma insulin (PI) was estimated by ultra sensitive rat insulin kit (Crystal Chem, IL, USA) [16]. Systolic blood pressure (SBP) was recorded on the last day of the treatment in all groups using a tail cuff blood pressure recorder (AD Instruments, Australia) [17].

2.3. Vascular tissue experiments

After development of hyperinsulinemia and hyperglycemia, animals were sacrificed. The thoracic aorta (from the arch of aorta to the diaphragm) was quickly excised and placed in ice-cold oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit buffer (KHB). The aorta was cut into 5 mm segments after it was cleaned of adhering fat and adventitial tissues. This was freed from fat and connective tissue. Care was taken not to stretch the vessel. Aortic rings were made by cutting it with sharp micro scissors and were placed in 10 ml organ bath containing a modified Krebs Henseleit buffer (KHB) (NaCl – 119 mM, KCl – 4.75 mM, NaH_2PO_4 – 1.19 mM, $MgSO_4$ – 1.19 mM, $CaCl_2$ – 2.54 mM, $NaHCO_3$ – 25 mM, and glucose – 11 mM) of pH 7.4. The solution was continuously aerated with carbogen (95% O_2 + 5% CO_2) at 37 °C. Tissue was subjected to equilibration for 120 min under the tension of 2 g. The bath fluid was changed for every 15 min. Then, contraction was measured isometrically by using force transducer (77005F, UGO Basile, Italy). At the end of every experiment, tissue was subjected to drying. Then, dried weight of tissue was taken for the calculation of contraction in terms of tension [5,18].

2.4. Experimental design for vascular studies

Aortic ring was suspended by a pair of stainless steel hooks in water-jacketed organ bath [UGO Basile, Italy], filled with 10 ml of oxygenated Krebs-Henseleit buffer (KHB) maintained at 37 °C. A resting tension of 2 g was applied to the aortic rings, which were then allowed to equilibrate for 2 h and the buffer was changed every 15 min. The tissue was exposed to 80 mM KCl depolarizing solution. After two such challenges, cumulative contractile responses to increasing concentrations of Ang II (1 nM–30 μM) were recorded. Aortic rings were pre-contracted with sub-maximal concentration of phenylephrine (PE, 100 nM) to evaluate acetylcholine (1 nM–30 μM) induced vasodilatation [3,18]. The responses were normalized to cross sectional area of the tissue and the tension developed was calculated as described before [3,5,18].

2.5. Immunohistochemistry

Immunohistochemistry was performed as per the protocol described by Gaikwad et al., [17]. Briefly, from each rat, portion of aorta tissue was fixed in 10% (v/v) formalin in phosphate buffer solution (PBS) and embedded in paraffin after completing the routine processing. For immunohistochemistry, 5 μm sections were taken from paraffin blocks and deparaffinised with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L, pH-6). The following primary antibodies were used: anti- Angiotensin II type 1 receptors (AT_1R), anti- Angiotensin II type 2 receptors (AT_2R), anti- Angiotensin-converting enzyme 2 (ACE2) (rabbit, 1:50 dilution) (Santa Cruz Biotechnology, CA, USA), anti-kelch ECH Associated Protein 1(Keap1), anti- Transforming growth factor-beta (TGF-β) (rabbit, 1:50 dilution) (Cell Signaling Technology, MA, USA) and HRP linked anti-rabbit secondary antibody was used, followed by detection with diaminobenzidine (DAB) as a chromogen. Slides were counterstained with haematoxylin, dehydrated by using alcohols and xylene, and mounted in DPX (Sigma Aldrich). For each group, at least 25 aorta sections were observed under microscope (Olympus BX41, NY, USA) and images were captured. The intensity of the spots was graded from 1 to 4 (1, slight or no color; 2, very low color; 3, moderate brown color; and 4, very intense brown color) [18]. The immunohistochemistry score is expressed as means ± S.E.M. for each experimental group.

2.6. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison between different groups was performed using one way analysis of variance (ANOVA) and if F value was significant

then multiple comparison was done by Tukey test using Prism software (version 5.0; GraphPad, San Diego, CA) for Windows. Cumulative concentration response curves were analyzed for pD2 value (the negative log concentration required to produce 50% of the maximal response) and maximal contraction (Emax), and statistical differences between the means were determined by one-way ANOVA followed by Tukey test. Immunohistochemical scores were analyzed using Kruskal–Wallis ANOVA on ranks, followed by the Tukey test. Data was considered statistically significant if $p < 0.05$.

3. Results

3.1. Esculetin attenuates metabolic alterations in hyperinsulinemia and hyperglycemia

Fasting plasma glucose (PGL), triglycerides (PTGs), and total cholesterol (PTC) was increased in HFD and HFD + STZ treated rats as compared to normal control (NC), which was significantly reduced by esculetin treatment (Table 1). Further, the plasma insulin level was increased in HFD and decreased in HFD + STZ treated rats, and esculetin alleviates these alterations in insulin level under hyperinsulinemic condition, more effectively at 100 mg/kg/day dose (Table 1). Esculetin and STZ treated normal rats did not show any significant changes in these parameters as compared to NC rats (Table 1). These results suggest that esculetin is able to improve the insulin sensitivity under these two conditions.

3.2. Esculetin reduces systolic blood pressure and alleviates change in body weight

Systolic blood pressure (SBP) was raised in HFD and HFD + STZ treated rats, and increase in SBP was more pronounced in HFD + STZ treated rats. Esculetin treatment efficiently reduced this rise in SBP in both groups (Table 1). At the end of the study, body weight of all the animals was measured. Body weight of HFD animals was considerably higher and in contrast body weight of HFD + STZ group animals was markedly low as compared to NC animals. Interestingly, esculetin treatment effectively normalized the changes in body weight in both (hyperglycemic and hyperinsulinemic) conditions (Table 1). Esculetin more effectively reduces SBP and alleviates changes in body weight at 100 mg/kg/day dose. All these parameters were found close to NC in esculetin and STZ treated normal rats (Table 1), therefore these 3 groups were omitted in further evaluation.

3.3. Esculetin attenuates vascular hyper responsiveness to Ang II and impaired acetylcholine mediated relaxation

The cumulative concentration response curves (CRCs) to Ang II in aortic ring preparations were obtained, and as depicted in Fig. 1A. CRCs of HFD and HFD + STZ treated diabetic rats showed an upward shift indicating an increase in maximal contractile response to Ang II challenge in comparison to NC rats. Esculetin significantly attenuated the exaggerated vascular responsiveness to Ang II in both the conditions. In addition, HFD + STZ treated diabetic rats showed impaired acetylcholine mediated relaxation, which was significantly alleviated by esculetin treatment Fig. 1B. There was no significant difference among all the groups in terms of the pD2 value, indicating that the sensitivity of aortic rings from different groups to Ang II remain unchanged.

3.4. Esculetin prevents alterations in renin-angiotensin system (RAS) and reduces TGF-β and Keap1 expression in hyperinsulinemia and hyperglycemia

The AT_1R and AT_2R expressions were significantly increased in aorta of HFD and HFD + STZ treated animals, while the ACE2 expression was significantly decreased in HFD + STZ treated animals compared to NC rats (Fig. 2A–C). Interestingly, esculetin treatment reduces AT_1R, and AT_2R protein level and increases ACE2 protein level in both the conditions (Fig. 2A–C). In addition, to assess the effect of esculetin treatment on the vascular fibrosis and oxidative stress, we examined levels of TGF-β and Keap1 marker of fibrosis and oxidative stress, respectively, in the aorta (Fig. 2D–E). The level of TGF-β and Keap1 protein expression was found to be augmented in aorta of HFD and H + S treated rats and esculetin treatment prevented these changes (Fig. 2D–E).

4. Discussion

The aim of this study was to investigate the protective effect of esculetin (6,7-dihydroxy coumarin) on altered vascular reactivity associated with hyperinsulinemia and hyperglycemia. Following findings provided convincing evidence of the beneficial effect of esculetin on vascular tissue in diabetes-esculetin (a) prevents metabolic perturbations, (b) inhibits rise in systolic blood pressure, (c) attenuates exaggerated vascular responsiveness of thoracic aorta to Ang II, (d) alleviates altered vascular reactivity to acetylcholine challenges, and (e) attenuates changes in AT_1R, AT_2R, ACE2, TGF-β, and Keap1 expression in aorta associated with hyperinsulinemia and hyperglycemia. The present study unveils the

Table 1
Effect of esculetin treatment on metabolic alteration in insulin resistance and type 2 diabetic rats.

Group	Plasma glucose (mmol/l)	Plasma triglycerides (mg/dl)	Plasma total cholesterol (mmol/l)	Plasma insulin (pmol/ml)	SBP (mmHg)	Body weight (g)
Normal control **(NC)**	5.75 ± 0.22	42 ± 2.24	1.53 ± 0.02	2.35 ± 0.07	87.67 ± 0.49	165 ± 1.85
Normal control/STZ (35 mg/kg) **(NC + S)**	5.91 ± 0.15	44 ± 2.18	1.54 ± 0.11	2.47 ± 0.19	86.52 ± 0.23	166 ± 5.27
Normal control/esculetin (50 mg/kg) **(NC + E1)**	5.65 ± 0.29	42 ± 4.33	1.51 ± 0.07	2.24 ± 0.15	85.69 ± 0.58	165 ± 2.51
Normal control/esculetin (100 mg/kg) **(NC + E2)**	5.98 ± 0.24	42 ± 2.49	1.67 ± 0.11	2.22 ± 0.08	84.52 ± 0.29	167 ± 2.75
High fat diet control **(HFD)**	6.78 ± 0.08*	61 ± 2.13*	4.01 ± 0.10*	6.24 ± 0.44*	97.50 ± 0.50*	194 ± 3.77*
High fat diet/esculetin (50 mg/kg) **(H + E1)**	5.91 ± 0.20$^{\#}$	51 ± 2.44	2.98 ± 0.12$^{\#}$	6.55 ± 0.50$^{\#}$	93.50 ± 0.84	193 ± 2.55$^{\#}$
High fat diet/esculetin (100 mg/kg) **(H + E2)**	5.69 ± 0.30$^{\#}$	45 ± 1.82$^{\#}$	2.67 ± 0.06$^{\#}$	2.61 ± 0.12$^{\#}$	88.00 ± 0.51$^{\#}$	180 ± 3.39$^{\#}$
High fat diet/STZ (35 mg/kg) **(H + S)**	23.68 ± 0.16*	156 ± 10.87*	11.11 ± 0.38*	2.08 ± 0.10*	116.0 ± 0.63*	117 ± 4.92*
High fat diet/STZ (35 mg/kg)/esculetin (50 mg/kg) **(H + S + E1)**	17.89 ± 0.61$^{\$}$	110 ± 3.09$^{\$}$	6.68 ± 0.41$^{\$}$	3.02 ± 0.15$^{\$}$	101.7 ± 1.11	120 ± 3.11$^{\$}$
High fat diet/STZ (35 mg/kg)/esculetin (100 mg/kg) **(H + S + E2)**	8.26 ± 0.62$^{\$}$	54 ± 3.38$^{\$}$	3.00 ± 0.31$^{\$}$	3.94 ± 0.19$^{\$}$	88.33 ± 0.91$^{\$}$	157 ± 5.36$^{\$}$

Note: Each data is represented as mean ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs H + S].
Bold letter in the table indicates abbreviated form of the group used in the study.

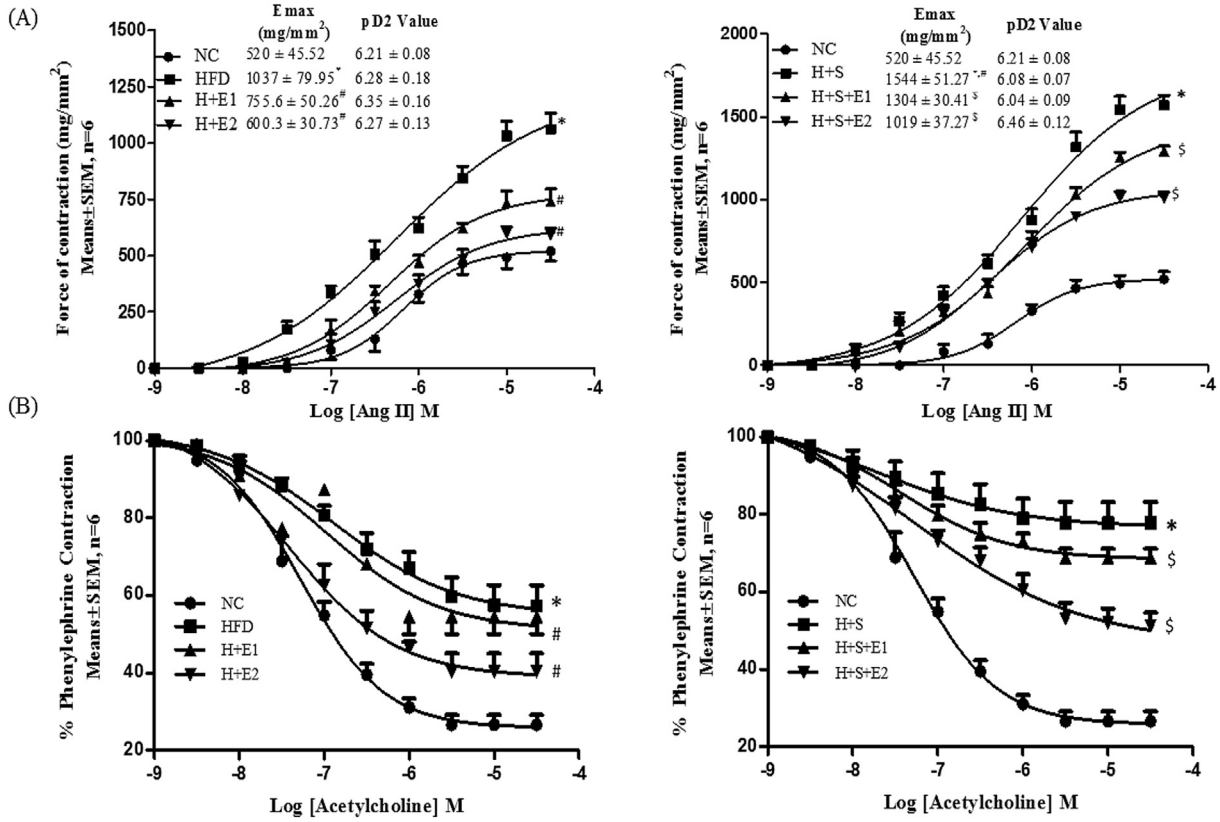

Fig. 1. Effect of esculetin on Ang II and Acetylcholine mediated vascular reactivity. Cumulative CRCs to (A) Ang II and (B) acetylcholine in aortic rings obtained from NC, HFD (left panel), and H + S (right panel) fed rats treated with esculetin (n = 6 rats/group). Note: (*) vs NC; (#) vs HFD; ($) vs H + S.

protective effect of esculetin on vascular dysfunction associated with IR and type 2 diabetes.

In this study, hyperinsulinemia and hyperglycemia were developed by HFD feeding and administration of low dose of STZ (35 mg/kg), which was confirmed by increased plasma insulin (PI) and plasma glucose (PGL) level in HFD and HFD + STZ treated rats, respectively. In this non-genetic model, animals exhibited the metabolic and subsequent pathophysiological alterations which were similar to that of human type 2 diabetes. Hyperinsulinemia and hyperglycemia were associated with significant rise in SBP (hypertension) and altered plasma lipid profile [5], which were attributed, at least in part, to vascular dysfunction. Esculetin treatment alleviated metabolic alterations, and normalized plasma lipid profile in both the conditions, these findings are in harmony with earlier study results obtained in animal model of STZ induced diabetes [6–8,19]. In addition, esculetin treatment abrogated the elevations in SBP in both the conditions.

The study was mainly focused on altered vascular reactivity, because it is considered as hallmark of diabetic vascular complications. Accumulated body of evidence suggests that, hyperinsulinemia and hyperglycemia can modulate the physiological responses to Ang II, which may contribute to the pathogenesis of vascular dysfunction associated with diabetes [5,20]. The thoracic aorta isolated from HFD and HFD + STZ treated rats showed exaggerated vasoconstriction responses to Ang II but the sensitivity (pD2 value) remain unchanged, which are consistent with previous reports [4,5]. Ang II- an extremely potent hormone of RAS modulates various deleterious actions through AT_1R. Over activation of RAS and increased Ang II level result into increased oxidative stress and uncoupling of the endothelial nitric oxide synthase (eNOS) which largely account for vascular dysfunctions [21,22]. Ang II induced exaggerated contraction of aorta was significantly attenuated by esculetin treatment in both the conditions. In the vascular tissues, acetylcholine stimulates production and release of endothelial derived relaxing factors (e.g. nitric oxide and prostacyclin), and produce endothelium dependent and nitric oxide mediated relaxation [23,24]. In addition, aortic ring of HFD and HFD + STZ treated rats showed impaired endothelium dependent relaxation to acetylcholine challenge as compared to normal control rats. Hyperinsulinemia and hyperglycemia causes tissue damage by several mechanisms including oxidative stress, increased advanced glycation end products (AGEs) formation, and apoptosis which might be the contributing factors for impaired endothelium dependent relaxation and vascular dysfunctions [25,26]. It has been reported that, an AMPK activator metformin attenuated impaired acetylcholine induced endothelial dependent relaxation in aorta of HFD fed mice, and recently AMPK modulating effect of esculetin has been reported [13,14]. Our results showed that, esculetin treatment improves endothelial dependent relaxation of aortic rings in both the conditions, which might be, in a part, contributed to its protective effect on vascular dysfunctions.

To investigate molecular mechanisms behind protective effect of esculetin on vascular dysfunction in hyperinsulinemia and hyperglycemia, we checked the expression of AT_1R, AT_2R and ACE2. It has been reported that, in diabetes AT_1R and AT_2R were increased and ACE2 expression was decreased in aorta [4,18,27,28]. In the present study, we also observed similar changes in the aorta of hyperinsulinemic and hyperglycemic rats. Besides RAS, TGF-β also plays an important role in the extracellular matrix accumulation and vascular remodeling by up-regulating connective tissue growth factor (CTGF) and fibroblast growth factor [29,30]. Vidagliptin

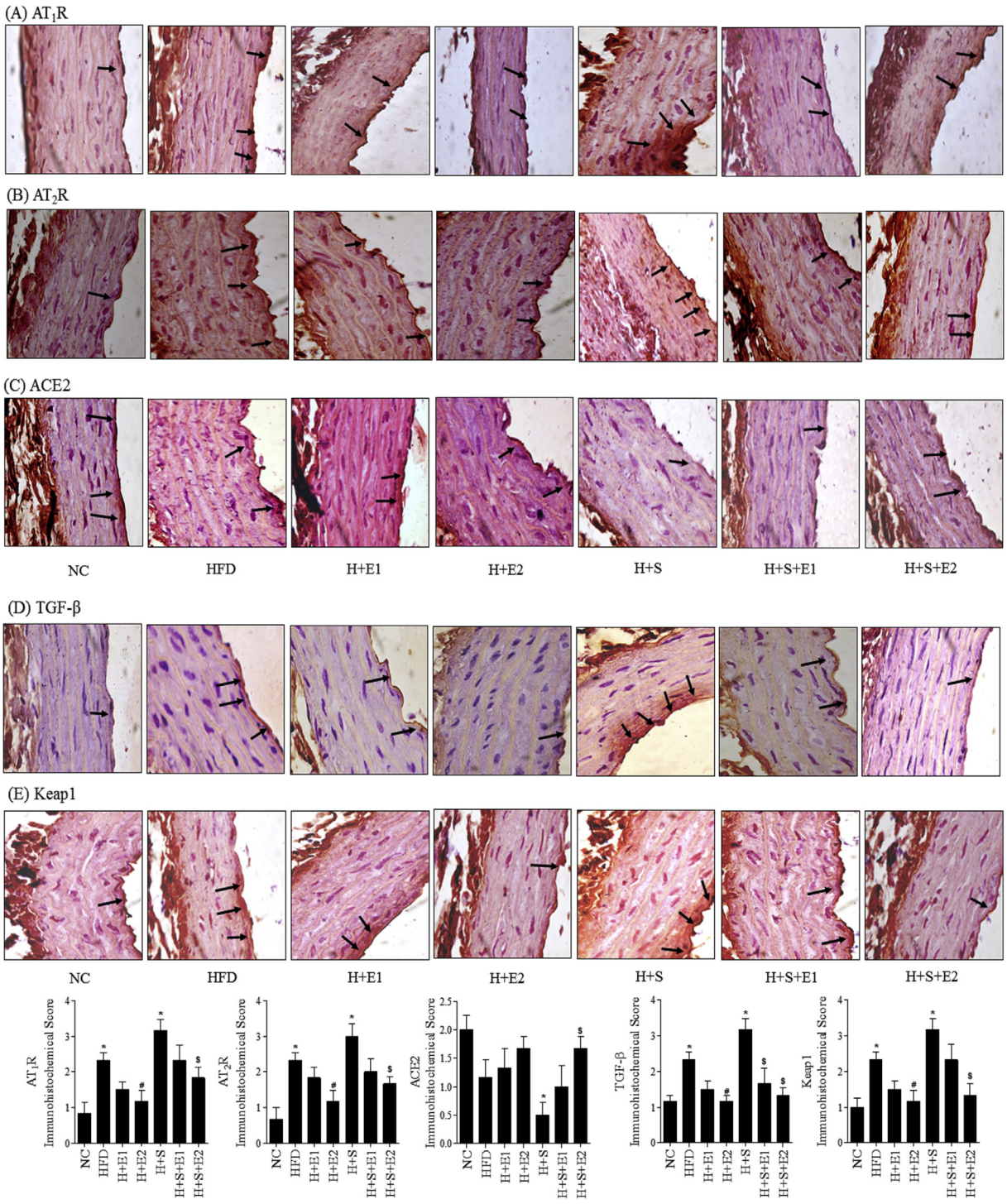

Fig. 2. Effect of esculetin on AT_1R, AT_2R, ACE2, TGF-β and Keap1 expression in hyperinsulinemia and hyperglycemia. From each group representative image was shown (original magnification, × 100 and Scale bar- 50 μm). Semi-quantitative analysis was done as describe in method section and immunohistochemistry score is represented as mean ± SEM, n = 6 rats/group. Note: (*) vs NC; (#) vs HFD; ($) vs H + S.

(dipeptidyl peptidase-IV inhibitor) found to be protective against vascular injury in diabetes by reducing TGF-β expression and attenuating the deleterious effects of AGEs [31]. In addition, HFD fed rats treated with Oltipraz (a nuclear respiratory factor 2 (NRF2) activator) restored impairment of the endogenous redox system (decrease in NRF2 and increase in Keap1) which is important in the development of obesity and insulin resistance [32]. Our results also show increased expression of TGF-β and Keap1 in the aorta of diabetic rats, which was significantly reduced by esculetin treatment, hence improving the insulin resistance and vascular dysfunction in diabetes.

Conflicts of interest statement

We declare that we have no conflict of interest.

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