Influence of pentoxifylline on gene expression of PAG1/ miR-1206/ SNHG14 in ischemic heart disease

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ABSTRACT

The regulation by immune checkpoint is able to prevent excessive tissue damage caused by ischemia reperfusion (I/R); therefore, the study aims to investigate the behavior of phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG1) mRNA, miR-1206 and small nucleolar RNA host gene 14 (SNHG14) during I/R and intake of pentoxifylline (PTX) as a protective drug. The relative expression level of PAG1/miR-1206/ SNHG14 was determined by qRT-PCR. Cardiac tissue levels of cytotoxic T-lymphocyte associated antigen 4 (CTLA4) and PAG1 protein expression were determined by ELISA technique. The regulatory T cells achieved by the flow cytometry. The results found that the relative expression of SNHG14 was significantly upregulated in I/ R, but suppressed in PTX treated groups with enhancement of the relative expression level of miR-1206. The gene and protein expression of PAG1 were downregulated with effective doses of PTX. The results showed that (30 and 40 mg/kg bwt) PTX dose suppressed the CTLA4 development significantly. The mean of the regulatory T cell in PTX protective groups is significantly reduced at ($p < 0.001$) in a comparison with I/R group. Spearman's correlation analysis revealed a significant negative correlation between SNHG14 and miR-1206, but a significant positive correlation between SNHG14 and PAG1 in I/R heart tissue. The results indicated that miR-1206 and SNHG14 can be used as biomarkers with perfect sensitivity and specificity. Using PTX reduced cardiac tissue damage. SNHG14 and miR-1206 can be used as a diagnostic tool in I/R.

1. Introduction

Several million deaths are attributable to ischemic heart disease (IHD), and the rate of recurrent ischemic events remains high even while providing treatment [1]. Leukocytes play an important role in the no-reflow phenomenon (a process which starts during the ischemic period and then increases during reperfusion) under pathophysiological conditions [2], resulting in profound myocyte death and irreversible myocardial damage [3]. The ischemic left ventricular dysfunction is characterized by the inflammatory immune activation [4]. Reperfusion is the only way to preserve cardiac cell viability, but it can trigger a further damage to the cardiomyocytes [5]. Discovery of early diagnostic markers of ischemic heart disease was widely investigated for reducing infarction size and mortality rate after I/R injury [6].

Pentoxifylline (PTX) is a phosphodiesterase inhibitor which has anti-inflammatory properties [7]. PTX and its metabolites increase the blood fluidity by reducing blood viscosity, inhibiting microvascular construction and blocking erythrocyte [8]. PTX regulated the inflammatory response with the decreased cellular activation, phagocytosis and endothelium adhesion [9]. Moreover, it was found to be a successful treatment for ischemic-like diseases such as arteriosclerosis obliterans (ASO) and Buerger's disease [10].

The phosphoprotein associated with glycosphingolipid microdomains (PAG1) is a transmembrane adaptor protein localized to lipid rafts [11]. PAG1 plays an important role in antigen trafficking and immune signaling [12]. In addition, northern blot analysis revealed that the highest expression was observed in lung, heart, and placenta [13]. The previous studies suggested that the increased expression of PAG1

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could have a proinflammatory impact [14]. Chromosome 8 data provide a deeper understanding of both the physiology of normal disease and the evolution of the genome; as it includes a variety of genes linked to innate immunity [15]. As PAG1 gene and miR-1206 were mapped to chromosome 8, they may be in association during the immune response.

The functional position of microRNA-1206 encoded on chromosome 8q24 is still elusive [16]. It is one of six annotated miRNAs found in the non-coding PVT1 locus [17]. PVT1 was found to be essential in the development and incidence of sepsis-induced inflammation and myocardial injury [18]. PVT1 and the miR-1204 cluster control may be linked to innate and adaptive immunity functions [19]. Recent studies have indicated that certain micro-RNAs and long non-coding RNA (lncRNA) can correlate with cardiac diseases, and may also be used as diagnostic and prognostic markers [20].

Various lncRNA interact with miRNAs and affect mRNA stability by masking miRNA responsive elements or by competing miRNA binding in competing endogenous RNA (ceRNA) networks [21]. Small nucleolar RNA host gene 14 (SNHG14), a novel lncRNA located on chromosome 15q11.2 in humans [22], was expressed within different analyzed tissues, including heart tissue [23]. It has been indicated that SNHG14 could act as a sponge for many microRNAs in different tissues affecting the cell apoptosis, proliferation and autophagy [24].

The aim of this study is to make use of PTX as a preventive drug in I/R and to investigate its impact on gene expression changes. Also, the study traces the competing endogenous RNAceRNA targeting immune checkpoint gene (PAG1) in ischemic heart developments and progression, followed by an examination of the correlation between SNHG14, miR-1206 and PAG1 in I/R of heart tissue during intake of PTX in different doses. Moreover, the gene expression signatures of PAG1, miR-1206 and SNHG14 in cardiac tissues will be studied for their novel utility as novel diagnostic and prognostic markers for I/R disease. As the expression level of miR-1206 in myocardial diseases has not yet been identified, the study aims to detect its expression level and follow its correlation with other biomarkers.

2. Materials and methods

Pentoxifylline was purchased from Sigma-Aldrich Company. Detection Kit of (CD4/CD25/FoxP3) (PE) 130-094-163 & (APC) 130-094-158, were supplied from Miltenyi Biotec.

2.1. Biomarker retrieval

Bioinformatics analysis was done to retrieve promising biomarkers relevant to I/R disease. The protein atlas database showed predicted relation between I/R disease and PAG1. (https://www.ebi.ac.uk/gxa/query?geneQuery=%5B%7B%22value%22%3A%22T%20cell%20receptor%20signaling%20pathway%22%2C%22category%22%3A%22%22%7D%5D&organism=homo%20sapiens&conditionQuery=%5B%7B%22value%22%3A%22ischemia%20reperfusion%22%7D%2C%7B%22value%22%3A%22heart%22%7D%5D&bs=%7B%22homo%20sapiens%22%3A%5B%22ORGANISM_PART%22%5D%7D&ds=%7B%22kingdom%22%3A%5B%22animals%22%5D%7D#differential).

The Prediction of miRNA Targets studied by using TargetMiner database (https://www.isical.ac.in/~bioinfo_miu/final_html_targetminer/hsa-miR-1206.html) and (http://signalink.org/protein/Q9NWQ8).

The target miRNA of SNHG14 was predicted using the DianaTools program (http://diana.imis.athena-innovation.gr/DianaTools/index.php). Also the lncRNA (Lnc_SNHG14, ENST00000549804) and miR-1206 acting as a competing endogenous RNAs available at (http://gyanxet-beta.com/lncedb/index.php).

Ethical approval

All procedures for the care and use of laboratory animals were approved by the Institutional Animal Ethics Committee for Cairo University, Faculty of Science (Ethical committee approval number: **CUI F6418**). Adult male wistar rats were obtained from Vaccine and Immunity Organization, Helwan, Egypt, and were accommodated in an animal house before the start of the experimental work, (an adaptation of one week).

2.2. Experimental design

Thirty adult male Wistar rats weighing 200 ± 20 g, were divided after the period of adaptation into five main groups, each group is composed of six rats. The first group was served as a naive. The second group was served as I/R group. The third, fourth and fifth groups, were injected with (PTX doses 20, 30, and 40 mg/kg bwt), respectively. PTX dose was injected 5 min before a 45 min occlusion of the left ventricle, followed by a 120 min reperfusion in rats. Rats were anesthetized by intraperitoneal injection with urethane (1.2 g/kg, i.p.) dissolved in water to apply the surgery. Anesthetized Wistar rats were intubated with a rodent ventilator at 70–80 breaths/min. The heart was exposed by median sternotomy and ligature was placed around the left anterior descending coronary artery. The free ends of the ligature were used to form a noose around a syringe plunger which was placed flat on the myocardium. Coronary occlusion was achieved by tightening the noose around the rubber band for 45 min [25]. The naive animals were exposed to the same procedure with no ligation reperfusion for 120 min. At the end of reperfusion, the animals were euthanized and the blood samples were collected and centrifuged for 10 min at 3000 r.p.m. to obtain serum for clinical measurement. Hearts were removed immediately, washed in saline (NaCl) solution 0.9% then collected and preserved for histopathology and molecular analysis.

2.3. Histopathological examination

After euthanasia heart was dissected out and fixed in 10% neutral formalin saline for 24 h, the tissue was washed, processed in different grades of alcohol, cleared in xylene and embedded in paraffin wax. 5 μm Sections were cut and routinely stained by hematoxylin and eosin (H&E) according to Ref. [26]. Slides were examined under light microscope (Olympus BX43) connected to a digital camera (Olympus DP 27) and images were received into the connected software (CellSens dimensions).

Histological scoring was performed according to Ref. [27]. Briefly, score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with presence of contraction bands and neutrophil infiltrate; score 4 (highly severe), widespread necrosis with presence of contraction bands, neutrophil infiltrate, and hemorrhage.

2.4. Biochemical analysis

Evaluation of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) enzymes were performed by (Spectrophotometer Unico® 1200) according to diagnostic kits obtained from Spectrum company, Cairo, Egypt.

2.5. ELISA assay of CTLA4 and PAG1 protein in cardiac tissue

Rat cardiac tissue levels of PAG1 and CTLA4 proteins were determined by an enzyme-linked immunosorbent assay according to the manufacturer's instruction of (SUNLONG BIOTECH Co., LTD. (China). Briefly, standards or samples were added and combined to the pre-coated wells with specific antibody. Then, a Horseradish Peroxidase (HRP) conjugated antibody specific for the protein was added to the wells and incubated. Finally, the enzyme-substrate reaction was terminated and the OD values were measured by (Sunostik ELISA reader). The concentration of PAG1 or CTLA4 protein in the samples was determined

and multiplied by the dilution factor.

2.6. Detection of CD4$^+$CD25$^+$FoxP3$^+$ Treg. cells in cardiac tissues by flow cytometry according to [28,29]

Cardiac tissue was fully digested to a cell suspension by adding digestive media. The tissue was minced in 10 ml of RPMI 1640 medium with 10% fetal calf serum, 600 U/ml collagenase II and DNase I. This mixture was shaken and the supernatant was filtered through a 100 μm nylon cell strainer. The suspension was washed several times in HBSS medium for 10 min.

Nucleated cells were re-suspended in buffer, then CD4-FITC and CD25 antibody were added in equal volumes, and wells were incubated in the refrigerator. Cells were washed by adding buffer and the supernatant was aspirated completely. Nucleated cells were re-suspended in fixation/permeabilization solution, and then the nucleated cells were washed and re-suspended in cold permeabilization buffer after incubation for 5 min and addition of FcR Blocking reagent. The Anti-FoxP3 antibody was added after washing of incubated cells and pellet was re-suspended in a suitable amount of buffer for analysis by (BD Accuri C6) flow cytometry instrument.

2.7. Quantitative PCR analysis for measurement of PAG1 gene, miR-1206 and Lnc_SNHG14

The quantification of mRNA-PAG1 was amplified from the Hs_PAG1_1_SG QuantiTect Primer Assay, cat no: 249900, ID: QT00032095 and the QuantiTect SYBR Green PCR Kit cat no: 204141 (Qiagen, Germany). The Primer sequence Hs_ACTB_1_SG QuantiTect Primer Assay, cat no: 249900, ID: QT00095431 was used as housekeeper gene. All samples were analyzed using the 5 plex Rotor-Gene PCR Analyzer (Qiagen, Germany).

Total and miRNAs were extracted from cardiac tissues using a miR-Neasy Mini Kit; cat no: 217004 (Qiagen, Hilden, Germany) according to the manufacturer's protocol followed by reverse transcription of cDNA synthesized using miScript II RT Kit; cat no: 218161; (Qiagen, Hilden, Germany). The quantification of miRNA-1206 level was amplified from Hs_miR-1206_1 miScript Primer Assay; cat no: 218300, ID: MS00014175 and the miScript SYBR Green PCR Kit, cat no: 218073. The gene expression was normalized by the Hs_SNORD68_11 miScript Primer Assay, cat no: 218300, ID: MS00033712 as a housekeeper gene.

The quantification of Lnc-SNHG14 expression level was amplified from RT^2 qPCR Primer Assay for Human IPW, Primer Assay; cat no: 330701:, ID: LPH04422A and the RT^2 qPCR SYBR Green/ROX Master Mix Kit, cat no: 330520. The gene expression was normalized by the Hs_ACTB_1_SG QuantiTect Primer Assay, cat no: 249900, ID: QT00095431 as a housekeeper gene.

2.7.1. Calculation of molecular data
Data Analysis was done using the ΔΔCT method and an endogenous reference control for normalization purposes to calculate gene expression. Data were presented as fold change in expression and were calculated as $2^{(-\Delta\Delta CT)}$, where $\Delta CT = CT$ target gene $- \Delta CT$ reference gene, and $\Delta\Delta CT = \Delta CT$ Experimental sample $- \Delta CT$ calibrator.

2.8. Statistical analysis

The data were analyzed using the computer facility of the available statistical software packages of SPSS® software (Version 23, SPSS Inc., Chicago, IL). Biochemical data were expressed as means ± standard deviation (SD), followed by Tukey's post test with a one-way analysis of variance (ANOVA), while the molecular data were expressed as median and percentile followed Mann Whitney test for non-parametric variables. Chi-square (x^2) test was analyzed with Kruskal-Wallis H test for non-parametric variables. ROC curve was analyzed for molecular data to determine sensitivity and specificity of each variable.

3. Results

3.1. Effects on LDH, CK-MB cardiac enzymes, PAG1 protein and CTLA4

Statistical analysis revealed that there was a highly significant increase in the mean of serum LDH and CK-MB level in I/R group as compared with naive at ($p < 0.001$) as listed in (Table 1). In addition, there was no statistical significance between PTX groups treated with doses (30 mg/kg and 40 mg/kg) at ($p > 0.05$). Induction of I/R showed a significant elevation in the levels of CTLA4 when compared to the naive group. All treated groups showed a significant reduction in comparison to the I/R group as listed in (Table 1). The PAG1 protein was highly expressed in I/R and PTX (20 mg/kg) groups in a comparison with naive animals at ($p < 0.001$). Moreover, there was no statistical significance between PTX groups treated with doses (30 mg/kg and 40 mg/kg) at ($p > 0.05$) as presented in (Table 1).

3.2. Flow cytometry of T-regulatory cells

To determine the role of Treg (CD4$^+$CD25$^+$ FoxP3$^+$) in remodeling of I/R in heart tissues of rats, a comparison with PTX groups (Fig. 1) revealed that Treg (CD4$^+$CD25$^+$FoxP3$^+$) in I/R rats were significantly increased in mean value (8.33 ± 0.03)% compared with naive rats (5.29 ± 0.05)% at ($p < 0.001$). Also, the mean of Treg (CD4$^+$CD25$^+$ FoxP3$^+$) decreased again in PTX protective groups to (6.15 ± 0.05)% at ($p < 0.001$) in a comparison with I/R group.

3.3. The relative gene expression of PAG1, miR-1206 and Lnc_SNHG14 among different studied groups

As listed in (Fig. 2A) PAG1 expression in naive group ranged from 0.85 to 1.22 with median (0.99) and Percentiles range (0.91–1.12), while I/R group showed fold change ranging from 2.41 to 4.47 with median (3.59) and percentile range (2.99–4.04). The statistical significant difference between the two groups was p value < 0.021. In PTX (20 mg) treated group showed fold change ranging from 3.58 to 17.75 with median (6.61) and percentiles range (4.17–13.11) at p value < 0.110 at the same time PTX (20 mg) group still significant with respect to naive at p value < 0.021. In PTX (30 mg) treated group showed fold change ranging from 1.39 to 6.28 with median (4.83) and percentiles range (2.43–6.24) at p value < 0.773 at the same time PTX (30 mg) group still significant with respect to naive at p value < 0.021. In PTX (40 mg) treated group showed fold change ranging from 0.38 to 1.49 with median (0.43) and percentiles range (0.40–0.97) at p value < 0.021 at the same time PTX 40 mg group showed no significance with respect to naive group at p value < 0.248. On the other hand, there was no

Table 1
The biochemical activities of LDH, CK-MB, PAG1 protein and CTLA4 in different studied groups.

	Naive	I/R	PTX (20 mg/kg)	PTX (30 mg/kg)	PTX (40 mg/kg)
LDH (U/ L)	561.0 ± 86.4	2294.0 ± 599.9*^{ab}	1383.3 ± 208.4	1051.3 ± 91.5$^\sharp$	1041.0 ± 41.2$^\sharp$
CK-MB (U/L)	231.5 ± 47	1305.8 ± 105.2*^{ab}	774.8 ± 229.1*$^\sharp$	347.0 ± 49.5$^\sharp$	365.5 ± 156.5$^\sharp$
CTLA4 (pg/ml)	143.0 ± 58.3	502.5 ± 12.4*^{ab}	323.3 ± 76.5	218.8 ± 75.5$^\sharp$	237.5 ± 13.4$^\sharp$
PAG1 (pg/ml)	282.0 ± 59.9	2036.0 ± 344.9*^{ab}	2348.5 ± 461.9*^{ab}	640.5 ± 198.5$^\sharp$	317.0 ± 144.3$^\sharp$

Values are mean ± SD; number of animals = 6 rats/each group. One-way ANOVA followed by Tukey's comparison test. LDH = Lactate dehydrogenase, CK-MB= Creatine Kinase MB, PAG1 protein = Phosphoprotein associated with glycosphingolipid-enriched microdomains 1, CTLA4 = Cytotoxic T-lymphocyte associated antigen 4, IR= Ischemia Reperfusion, PTX= Pentoxifylline. *P < 0.001 compared to naive group. $^\sharp$P < 0.001 compared to IR group. ^{a} P < 0.001 compared to PTX-30 mg/kg dose. ^{b} P < 0.001 compared to PTX-40 mg/kg dose.
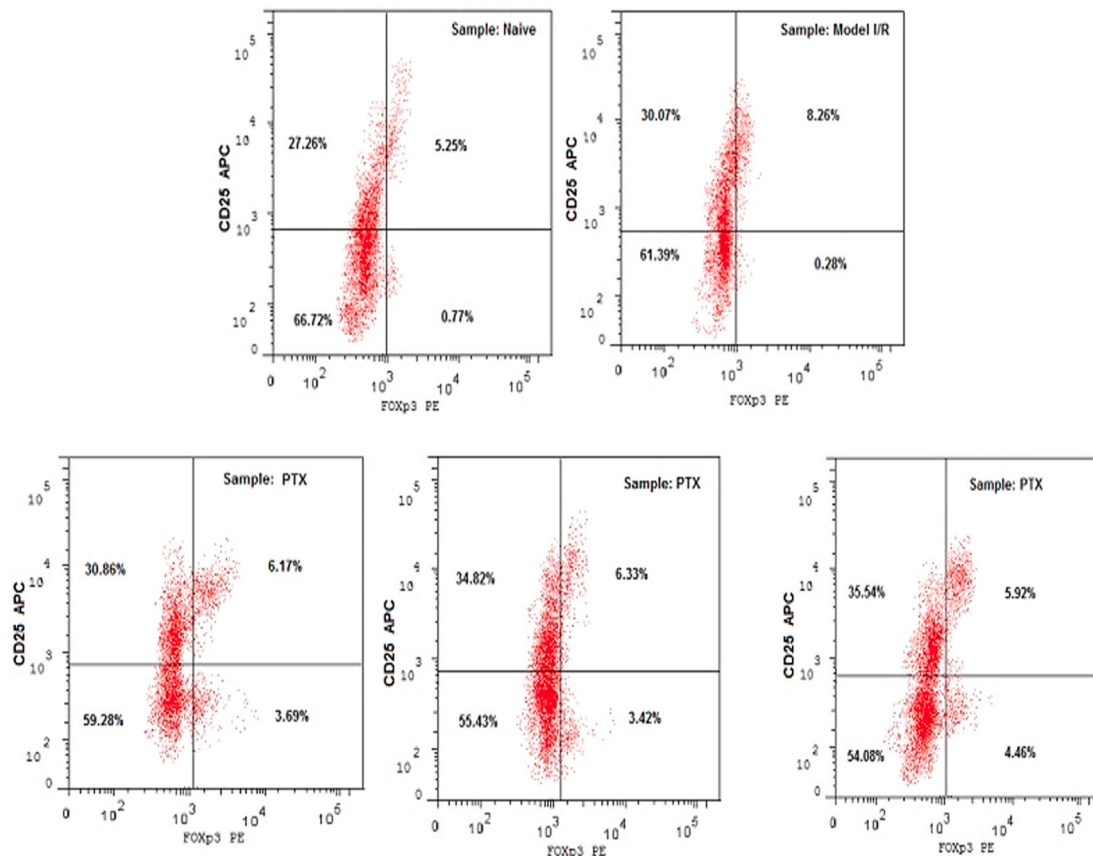
Fig. 1. Flow cytometry of T-regulatory cells in cardiac tissues.
Flow cytometry analysis of heart tissue samples from rats after treatment with either monoclonal CD25 antibodies conjugated to allophycocyanin (APC) and monoclonal Anti-FoxP3 antibodies conjugated to R-phycoerythrin (PE) to label all T lymphocytes. Antibodies to CD4 were conjugated with fluorescein isothiocyanate (FITC). Evaluation for statistical significance was performed and expressed as mean ± SEM. Differences were considered to be significant if (P ≤ 0.05); number of animals = 4 rats/each group. One-way ANOVA followed by Tukey's comparison test.

significant between PTX 20 mg treated group and PTX 30 mg at p value < 0.248, while PTX 40 mg treated group was significantly different from PTX 20 mg at p value < 0.021, and significantly different from PTX 30 mg at p value < 0.043. Chi square was analyzed with Kruskal-Wallis H test and showed a significant difference among the different groups of the study with p value < 0.006.

Expression of miR-1206 showed fold change with a statistically significant difference between naive and I/R group was (p < 0.020). PTX (20 mg/kg) group showed fold change with median (16.35 ± 7.5) at (p < 0.02), while PTX (30 mg/kg) group showed fold change with median (20.8 ± 8.3) at (p < 0.02). Moreover, PTX (40 mg/kg) group showed fold change with median (58.4 ± 11.6) at (p < 0.02). Chi square was showed a highly significant difference among the various research groups with (p < 0.001) as listed in (Fig. 2 B).

The fold change of SNHG14 in naive represented with median (1.14 ± 0.94), while the I/R group showed (4.66 ± 2.41) and there was a significant difference between them (p < 0.021). In PTX (20 mg/kg and 30 mg/kg) groups showed fold change with median (0.021 ± 0.041) and (0.007 ± 0.006) respectively at (p < 0.021). In PTX (40 mg/kg) the percent of reduction in median with respect to I/R (99.96%) at (p < 0.020) with fold change (0.002 ± 0.003) as showed in (Fig. 2C).

3.4. Correlation and ROC curve analysis for (PAG1) mRNA, miR-1206 and SNHG14

PAG1 showed a significant strong negative correlation with miR-1206 at (r = - 0.884, P ≤ 0.001) and a significant strong positive correlation with Lnc_SNHG14 at (r = 0.809, P ≤ 0.001). Furthermore,

Lnc_SNHG14 showed a significant negative correlation with miR-1206 at (r = - 0.739, P ≤ 0.006) as presented in (Fig. 3 A). The Treg.CD4/CD25/FoxP3 showed a significant strong correlation with PAG1 and miR-1206 respectively at (r = 0.692, P ≤ 0.013), (r = - 0.769, P ≤ 0.003). While there was no significant correlation with SNHG14 at (r = 0.371, P ≤ 0.235).

By using ROC curve as indicated in (Fig. 3 B and 3C), the sensitivity and specificity for PAG1, miR-1206 and SNHG14 were detected as shown in (Table 2), so the estimation of miR-1206 & Lnc_SNHG14 molecular parameter could be considered a perfect significant test in predicting ischemic heart disease.

3.5. Histopathological results

As shown in histopathology of I/R rats (Fig. 4) there was a massive myocardial destruction throughout the left ventricle with large areas coagulative necrosis and showed elevation number of inflammatory and infiltration cells. PTX treated groups showed significantly lower myocardial damage score comparing to the I/R rats. Histopathological score of myocardial lesions was listed in (Table 3). Diseased I/R group recorded the highest number in myocardial lesion score (3.7). PTX treated groups showed significantly lower sore comparing to I/R group.

**4. Discussion**

The ischemia period followed by reperfusion is associated with significant alterations in the transcription of gene expression which can help in diagnosis and treatment of disease. Moreover, reperfusion injury
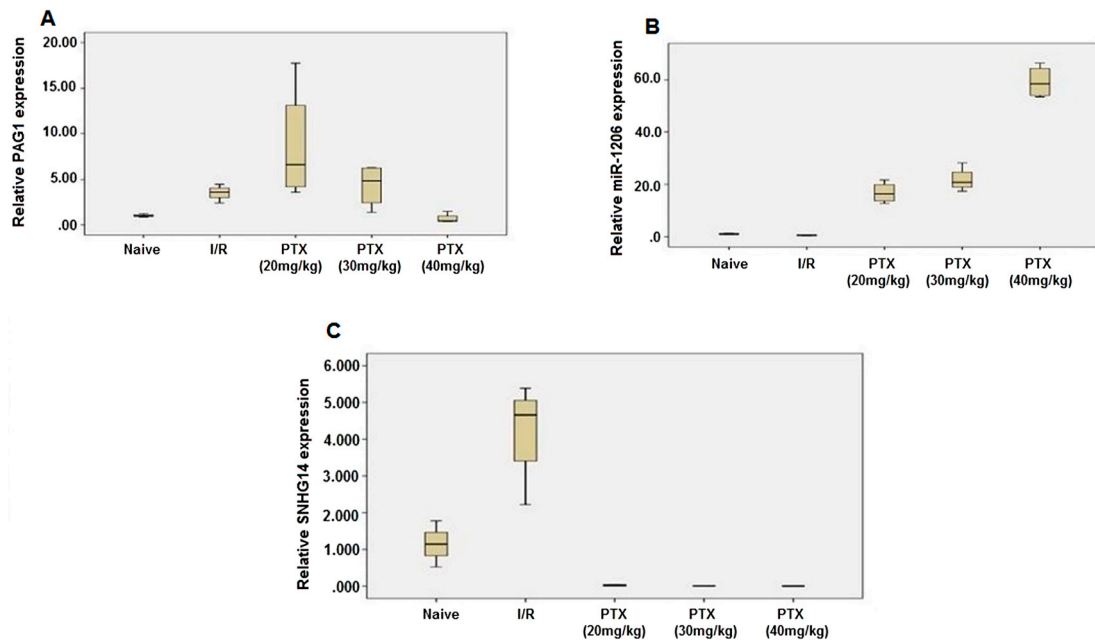
Fig. 2. Relative gene expression of molecular markers (PAG1, miR-1206, SNGH14) in different studied groups within heart tissues.
A) Gene expression of PAG1mRNA in different studied groups. B) Relative miR-1206 expression in different studied groups. C) Relative SNHG14 expression in different studied groups. I/R (Diseased group), Treated group: [PTX (20 mg/kg) + PTX (30 mg/kg) + PTX (40 mg/kg)], PTX= Pentoxifylline. Evaluation for statistical significance was performed and expressed as median ± Interquartile Range. Differences were considered to be significant if (P ≤ 0.05) followed Mann Whitney test for non-parametric variables. Number of animals = 6 rats/each group.
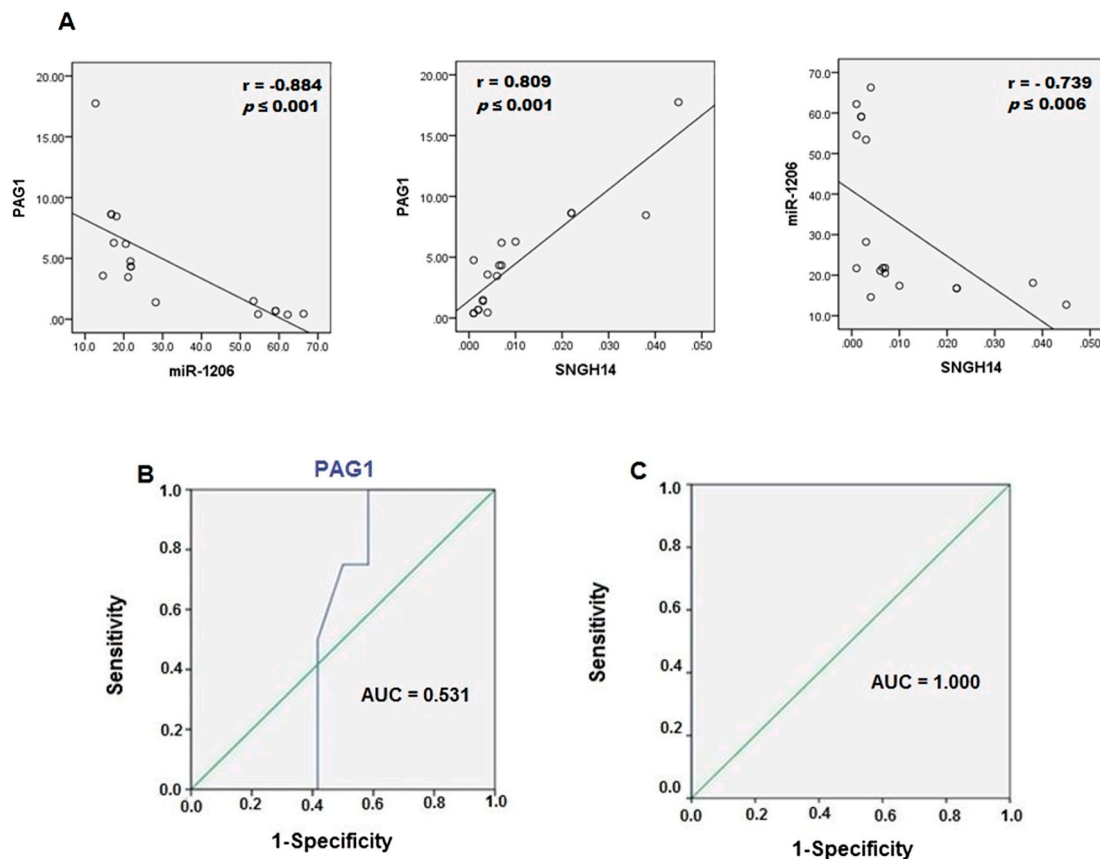
Fig. 3. Correlation and ROC curve analysis for PAG1, miR-1206 and SNHG14
A): Correlation curve for PAG1, miR-1206 and SNHG14 gene expression. B): Sensitivity and specificity of PAG1 gene expression. C): Sensitivity and specificity of miR-1206 and SNHG14. P > 0.05 was considered to be not significant, P ≤ 0.05 was considered to be statistically significant.

Table 2

Sensitivity, specificity, AUC, Cutoff value and p-values of the investigated markers.

|	Sensitivity	Specificity	AUC	Cutoff value	*p*-value
PAG1	100%	41.7%	0.531	1.95	0.856
miR-1206	100%	100%	1.000	6.7	0.004 *
Lnc_SNHG14	100%	100%	1.000	1.132	0.004 *

$P > 0.05$ was considered to be not significant, $P \leq 0.05$ was considered to be *Statistically significant.

Table 3

Histological score of myocardial lesion.

	Naive	I/R	PTX (20 mg/kg)	PTX (30 mg/kg)	PTX (40 mg/kg)
Myocardial lesion score	0.4 ± 0.2	3.71 ± 0.18	2.71 ± 0.28	2.14 ± 0.26	2.14 ± 0.26

Data are expressed as mean ± SEM. Histological lesion score of myocardium indicates significance in PTX groups in comparison with I/R group at $P \leq 0.05$.
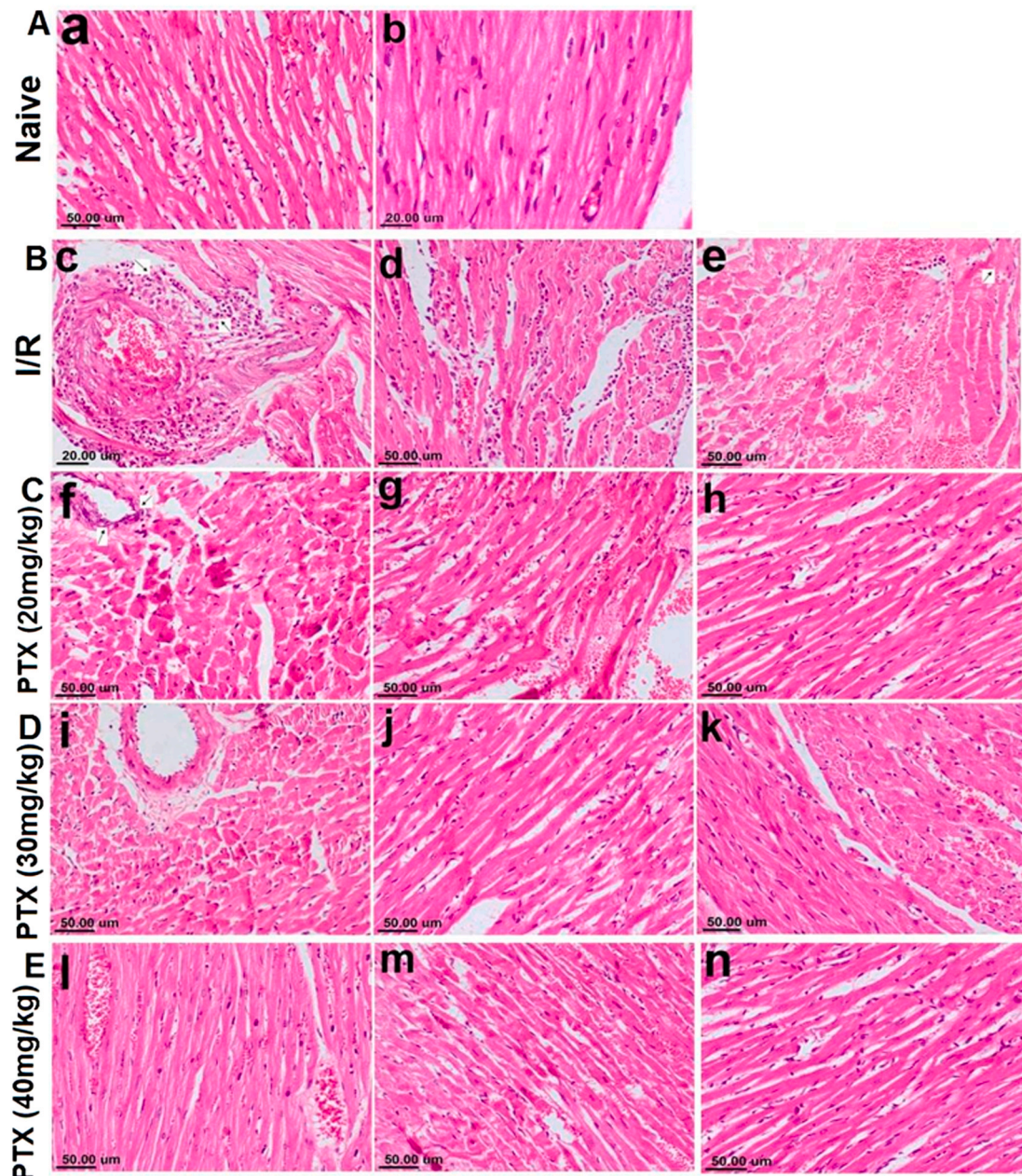
Fig. 4. Histopathology of heart tissues.

Photomicrograph of heart muscles, stained by H&E, of A) Naive group, showing normal structure of heart muscles. B) IR group, (c) showing perivascular edema and mononuclear inflammatory cells infiltration, (d) showing myocardial necrosis and inflammatory cells infiltration, (e) showing extensive myocardial necrosis, muscle fragmentation, hemorrhage with presence of contraction band necrosis (arrow). C) PTX (20 mg/kg) group, (f) showing degenerative changes and sporadic myocardial necrosis, (g) showing myocardial necrosis and hemorrhage, (h) showing apparently normal myocardium. D) PTX (30 mg/kg), (i) showing perivascular edema and necrosis of some cardiomyocytes, (j) showing apparently normal muscle fibers, (k) showing apparently normal muscle fibers with dilated blood vessels in between. E) PTX (40 mg/kg), (l) showing apparently normal myocardium with congestion, (m) showing coagulative necrosis of some muscle fibers with hemorrhage and (n) showing normal histological structure of myocardium.

leads to activation of innate and adaptive immune responses [30], so studying immune checkpoints to balance and control the immune response to prevent conversion to autoimmune disease is important. The number of T cells entering the heart within the first 30 min may contribute to an inflammatory cascade involving sequential activation of T cells and probably tissue-resident macrophages [31]. Some pharmacological agents are available to reverse the action of inflammation during I/R if it's used as a protective drug. Thus, the regulation of gene expression epigenetically by long non-coding and microRNA can be effective and helpful in understanding the therapeutic role.

PAG has microdomains which are rich in signaling molecules, such as Src-related protein tyrosine kinases (PTKs), G proteins, and receptors, and are approached to play an important role in cell signaling [32]. Analysis of the protein phosphatase association with PAG following T cell activation suggests that both cooperate with tyrosine kinase C-terminal Src kinase (CSK) to terminate T-cell activation. They primarily use PAG to control and overcome triggering of T-cell activation [33]. The critical cellular functions require that cytoplasmic protein CSK is recruited from the cytoplasm to the membrane by binding with PAG to enforce its negative regulatory effect by inactivating Src kinases to stabilize the protein tyrosine kinase Lck in inactive conformation supporting the PAG dephosphorylation. This is a crucial event during the initiation of T-cell activation [12] as well as PAG-overexpressing cells that have severe defects in TCR-induced proliferation [32]. PAG1 is considered as one of the immune checkpoint genes which acts as a negative T-cell regulator to suppress inflammation and control lymphocyte action.

Drugs that elevate intracellular cAMP levels are well-established as efficient regulators of innate and adaptive immunity by reducing the production of pro-inflammatory mediators and increasing the development of anti-inflammatory factors. These therapeutic strategies have immunoregulatory potential in autoimmune and inflammatory disorders [34]. PTX functions as a PDE inhibitor and induces prolonged levels of cAMP resulting in the activation of protein kinase A [35]. The cAMP/PKA is a potent negative regulator of T-cell receptor-mediated activation. In addition, it plays an important role in maintaining homeostasis in the T-cell and cellular structure of the heart through a proximal PKA/Csk/Lck-anchored pathway [36]. In this study, a negative-feedback loop might be working during T-cell activation to stabilize the activity of the enzymes responsible for cAMP degradation by increasing the local concentration of PDEs. The presence of PTX as a protective drug allows for the suppression of PAG1 production with effective doses; because of its role in regulating the immune system. Relatively overexpression of PAG1 was observed in this study to cause an inhibition of T-cell activation and to stabilize cellular membrane of heart tissues. Also, PTX may enhance the binding of Csk/PAG1 to inhibit T-cell activation.

The PAG1 levels correlate significantly with the expression of miR-1206, SNHG14 and Treg.CD4/CD25/FoxP3. The results show that there was a variation in the relative expression of the adaptor molecule PAG1 in the way of starting suppression during enhancement in treatment of pathological condition. The current study investigated the association between transmembrane adaptor protein and regulatory parameter during protective PTX intake for I/R of heart tissues; as it is found a suppression in PAG1 and SNHG14 relative expression level with enhancement of miR-1206 during intake of PTX as protective drug with effective doses, and this also modulates the stability of membrane.

To trace the link, we examined the relative expression levels of the predicted molecular regulatory elements: miR-1206 and lnc_SNHG14. We found that PAG1 may be connected to miR1206, as observed in different protective doses, which explains that miR-1206 may be act as RNA interference. This may predict that miR-1206 could play an essential role in the control of PAG1 gene and affect the immune system indirectly; as it is essential for homeostasis of T-cell function. In I/R diseased rats, during hypoxia, miR-1206 was downregulated while highly upregulated in different doses of cardioprotective drug. These

results can be used as signal for pharmacological approaches to conduct further studies on miR-1206 as a regulator of genes by silencing or enhancing during the ischemia reperfusion of the heart.

SNHG14 was previously found highly expressed in ischemic cerebral tissues followed by increased in apoptosis rate and increment in TNF-α [37], these data agree with the overexpression of SNHG14 during I/R of the heart in the present results. We found a significant difference in the expression in all parameters and we showed the diagnostic impact of the non-coding parameter.

A wide range of pathological processes contributed to ischemia and reperfusion associated-tissue injury, as hypoxia, is associated with an impaired endothelial cell barrier function due to the decreases in adenylate cyclase activity and intracellular cAMP levels, and the concomitant increase in vascular permeability and leakage [38]. Pharmacotherapy, which upregulates cAMP by inhibition of PDE activity, will prevent the development of CTLA4 and may enhance the function of regulatory T-cells [34]. This statement is compatible with our result as shown when PDE inhibitor drug (PTX) dose increased, the CTLA4 development was suppressed. Moreover, this allows Treg. cells to maintain an immunologic tolerance, and affects the activation and function of innate immune cells (e.g., macrophages, neutrophils and monocytes) to give a normal histological structure.

CTLA4 is an inflammatory adenosine signal that prevents immunological response, and protects the tissues from associated injury, tissue breakdown and hypoxia which generates high levels of extracellular adenosine [39]. Adenosine receptor (A2AR) mediated an increase in the population of $CD4^+$ $CD25^+$, then the increased $CD4^+$ cells expressed not only CD25 and FoxP3, but also CTLA4 [40]. Tregs also modulates the macrophage function; the predominant mechanism by which Tregs exerts its inhibitory effect on immune responses through the expression of CTLA4 molecule on its surface. CTLA4 binds to APC, and thereby prevents CD28-mediated activation of $CD4^+$ T-helper cells leading to downregulation of T-lymphocyte cytotoxic mediated inflammatory responses [41], and also enhancing FoxP3 expression [42]. Furthermore, Tregs are able to produce immunosuppressive cytokines, such as IL-10 and TGF-β, which decrease the risk of autoimmunity [43].

The present results indicate that protecting the heart is accompanied by a decrease in the infiltration and accumulation of Treg cells during reperfusion after myocardial ischemia by using different doses of PTX. Consequently, without down-regulating Treg cells, physiological processes are at high risk of becoming pathological. Thus, down-regulation is of great importance to allow immune responses to be completed [44]. Furthermore, we found a higher expression level of Foxp3 in PTX-treated groups. Foxp3 is important for the differentiation and suppression role of Treg cells [45], which is compatible with current data. Our existing data indicated that T-regulatory cells are activated during I/R and contribute to inflammatory cascade observed by infiltration of inflammatory cells in the heart tissue as shown in histopathology. These inflammatory cells may be activated due to the production of reactive oxygen species during the occlusion of left ventricle vein.

T regulatory cells can inhibit the immune response by number of mechanisms one of them through cAMP-mediated immunosuppression through a PKA/Csk/Lck pathway; A response for cAMP to activate PKA can effect on the forming of a complex with the Csk Binding Protein [46], so changing the regulatory T cell may be in relationship with the gene expression changes of PAG1/miR-1206/SNHG14. For this reason, we studied the Spearman's correlation between T-regulatory and PAG1; as they act as negative T-cell regulator to preserve immune tolerance. As the study revealed, the regulation of PAG1 may be affected with regulatory molecular elements (miR-1206 and Lnc_SNHG14). The detection of Treg.CD4/CD25/FoxP3 and gene expression changes under the effect of PTX doses showed a significant positive correlation with PAG1.

In addition, we found that the Tregs expression is correlated with miR-1206 significantly in PTX treated groups. These data indicate that miR-1206 may regulate some genes related to controlling immune

response and inflammation. In the present study, CTLA4, PAG1 and Treg cells are upregulated in I/R through the initial activation of T-cells within acute inflammation and contributed to preserving self-tolerance. We found that Treg and CTLA4 accumulated in injured heart tissues to contribute to the repairing processes. However, PTX treatment neutralizes Treg's suppressive function and downregulates CTLA4.

Moreover, the results presented herein that PTX with different doses is a good inducer of cardioprotection during I/R of heart; as the levels of LDH and CK-MB isoenzymes, which increased during I/R state, decreased significantly in the treated groups due to variation in membrane integrity. The results demonstrated that PTX remarkably improved the I/R-induced heart injury through inhibiting inflammation, and reducing the oxidative stress and the myocardial pathological damage.

PTX regulates immune system, as shown, we found that CTLA4, Treg cells and PAG1 help to prevent immunological response to prevent autoimmunity under the effect of PTX as a protective drug, and this was observed and confirmed in the light of a decreased inflammatory cell count in histopathology, an improved myocardial lesion score, a myocardial necrosis and muscle fragmentation, to finally give a normal histological structure.

5. Conclusion

The increase in PAG1 expression was correlated with the persistence of cardiac tissue damage, while the inhibition of PAG1 expression could be associated with the efficient dose of phosphodiesterase inhibitor drugs, such as PTX. Measuring the relative gene expression levels of miR-1206 and SNHG14 in cardiac tissue during I/R have a diagnostic role.

Author contributions

AS and ME studied concept and experimental design. AA and AMA: performed statistical analysis of experimental data. AA and MAE wrote the manuscript, and performed the molecular and biochemical experimental work. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

References

[1] R.J. Gryka, L.F. Buckley, S.M. Anderson, Vorapaxar: the current role and future directions of a novel protease-activated receptor antagonist for risk reduction in atherosclerotic disease, Drugs R 17 (2017) 65–72.

[2] S.H. Rezkalla, R.A. Kloner, No-reflow phenomenon, Circulation 105 (5) (2002) 656–662, https://doi.org/10.1161/hc0502.102867.

[3] C.M. Cao, Y. Zhang, N. Weisleder, C. Ferrante, X. Wang, F. Lv, Y. Zhang, R. Song, M. Hwang, L. Jin, J. Guo, W. Peng, G. Li, M. Nishi, H. Takeshima, J. Ma, R.P. Xiao, MG53 constitutes a primary determinant of cardiac ischemic preconditioning, Circulation 121 (2010) 2565–2574, https://doi.org/10.1161/CIRCULATIONAHA.110.954628.

[4] K. Sliwa, A. Woodiwiss, V.N. Kone, G. Candy, D. Badenhorst, G. Norton, C. Zambakides, F. Peters, R. Essop, Therapy of ischemic cardiomyopathy with the immunomodulating agent pentoxifylline results of a randomized study, Circulation 109 (2004) 750–755, https://doi.org/10.1161/01.CIR.0000112568.48837.60.

[5] M. Zhang, L. Yu, H. Zhao, X. Zhou, Q. Yang, F. Song, L. Yan, M. Zhai, B. Li, B. Zhang, Z. Jin, W. Duan, S. Wang, 2,3,5,4′ Tetrahydroxystilbene-2-O-β-D-glucoside protects murine hearts against ischemia/reperfusion injury by activating Notch1/Hes1 signaling and attenuating endoplasmic reticulum stress, Acta Pharmacol. Sin. 38 (2017) 317–330.

[6] H.K. Kim, S.W. Kang, S.H. Jeong, N. Kim, J.H. Ko, H. Bang, W.S. Park, T.H. Choi, Y. R. Ha, Y.S. Lee, J.B. Youm, K.S. Ko, B.D. Rhee, J. Han, Identification of potential target genes of cardioprotection against ischemia–reperfusion injury by express sequence tags analysis in rat hearts, J. Cardiol. 60 (2012) 98–110, https://doi.org/10.1016/j.jjcc.2012.02.004.

[7] D. Skudicky, A. Bergemann, K. Sliwa, G. Candy, P. Sareli, Beneficial effects of pentoxifylline in patients with idiopathic dilated cardiomyopathy treated with angiotensin-converting enzyme inhibitors and carvedilol results of a randomized study, Circulation 103 (2001) 1083–1088, https://doi.org/10.1161/01.CIR.103.8.1083.

[8] M. Aziret, O. Irkorucu, E. Reyhan, H. Erdem, K. Das, S. Ozkara, A. Surmelioglu, S. Sozen, I. Bali, S. Cetinkunar, K.C. Deger, The effects of vardenafil and pentoxifylline administration in an animal model of ischemic colitis, Clinics 69 (2014) 763–769, https://doi.org/10.6061/clinics/2014(11)10.

[9] M.E. Dávila-Esqueda, F. Martinez-Morales, Pentoxifylline diminishes the oxidative damage renal tissue induced by streptozotocin in the rat, Exp Diabesity 5 (4) (2004) 245–251.

[10] A.R. Akar, M.B. İnan, C. Baran, Thromboangiitis obliterans, Curr Treat Options in Rheum 2 (2016) 178–195, https://doi.org/10.1007/s40674-016-0047-6.

[11] M. Hrdinka, V. Horejsi, PAG - a multipurpose transmembrane adaptor protein, Oncogene 33 (2014) 4881–4892, https://doi.org/10.1038/onc.2013.485.

[12] A. Svec, Expression of transmembrane adaptor protein PAG/Cbp in diffuse large B-cell lymphoma: immunohistochemical study of 73 cases, Pathol. Res. Pract. 203 (2007) 193–198, https://doi.org/10.1016/j.prp.2007.01.005.

[13] T. Brdicka, D. Pavlistova, A. Leo, E. Bruyns, V. Korinek, P. Angelisova, J. Scherer, A. Shevchenko, I. Hilgert, J. Cerny, K. Drbal, Y. Kuramitsu, B. Kornacker, V. Horejsi, B. Schraven, Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase Csk and is involved in regulation of T cell activation, J. Exp. Med. 191 (2000) 1591–1604.

[14] C.T. Vicente, S.L. Edwards, K.M. Hillman, S. Kaufmann, H. Mitchell, L. Bain, D. M. Glubb, J.S. Lee, J.D. French, M.A.R. Ferreira, Long-range modulation of PAG1 expression by 8q21 allergy risk variants, Am. J. Hum. Genet. 97 (2015) 329–336, https://doi.org/10.1016/j.ajhg.2015.06.010.

[15] C. Nusbaum, T.S. Mikkelsen, M.C. Zody, S. Asakawa, S. Taudien, M. Garber, et al., DNA sequence and analysis of human chromosome 8, Letters nature 439 (2006) 331–335, https://doi.org/10.1038/nature04406.

[16] F. Conte, G. Fiscon, M. Chiara, T. Colombo, L. Farina, P. Paci, Role of the long non-coding RNA PVT1 in the dysregulation of the ceRNA- ceRNA network in human breast cancer, PLoS One 12 (2) (2017), e0171661, https://doi.org/10.1371/journal.pone.0171661.

[17] H.K. Kim, L. Prokunina-Olsson, S.J. Chanock, Common genetic variants in miR-1206 (8q24.2) and miR-612 (11q13.3) affect biogenesis of mature miRNA forms, PLoS One 7 (10) (2012), e47454, https://doi.org/10.1371/journal.pone.0047454.

[18] F. Feng, Y. Qi, C. Dong, C. Yang, PVT1 regulates inflammation and cardiac function via the MAPK/NF κB pathway in a sepsis model, Exp. Ther. Med. 16 (2018) 4471–4478.

[19] A. Bisio, V.D. Sanctis, V.D. Vescovo, M.A. Denti, A.G. Jegga, A. Inga, Y. Ciribilli, Identification of new p53 target microRNAs by bioinformatics and functional analysis, BMC Canc. 13 (2013) 552. http://www.biomedcentral.com/1471-2407/13/552.

[20] Y. Zhang, L. Zhang, Y. Wang, H. Ding, S. Xue, H. Qi, P. Li, MicroRNAs or long noncoding RNAs in diagnosis and prognosis of coronary artery disease, Aging and disease 10 (2) (2019) 353–366, https://doi.org/10.14336/AD.2018.0617.

[21] Y. Tay, L. Kats, L. Salmena, D. Weiss, S.M. Tan, U. Ala, et al., Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs, Cell 147 (2011) 344–357.

[22] L. Meng, R.E. Person, W. Huang, P.J. Zhu, M. Costa-Mattioli, A.L. Beaudet, Truncation of Ube3a-ATS Unsilences paternal Ube3a and ameliorates behavioral defects in the angelman syndrome mouse model, PLoS Genet. 9 (12) (2013), e1004039, https://doi.org/10.1371/journal.pgen.1004039.

[23] C.R. Galiveti, C.A. Raabe, Z. Konthyr, T.S. Rozhdestvensky, Differential regulation of non-protein coding RNAs from Prader-Willi Syndrome locus, Sci. Rep. 4 (2015) 6445, https://doi.org/10.1038/srep06445.

[24] X. Zhang, P. Zhao, C. Wang, B. Xin, SNHG14 enhances gemcitabine resistance by sponging miR- 101 to stimulate cell autophagy in pancreatic cancer, BBRC (Biochem. Biophys. Res. Commun.) 510 (2019) 508e514, https://doi.org/10.1016/j.bbrc.2019.01.109.

[25] E.N. Churchill, M.H. Disatnik, D. Mochly-Rosen, Time-dependent and ethanol-induced cardiac protection from ischemia mediated by mitochondrial translocation of varepsilon PKC and activation of aldehyde dehydrogenase2, J. Mol. Cell. Cardiol. 46 (2009) 278–284, https://doi.org/10.1016/j.yjmcc.2008.09.713.

[26] J.D. Bancroft, M. Gamble, in: Theory and Practice of Histological Techniques, Elsevier health sciences, 2008.

[27] R. Aneja, P.W. Hake, T.J. Burroughs, A.G. Denenberg, H.R. Wong, B. Zingarelli, Epigallocatechin, a green tea polyphenol, attenuates myocardial ischemia reperfusion injury in rats, Mol. Med. 10 (1–6) (2004) 55–62.

[28] H. Yagi, T. Nomura, K. Nakamura, S. Yamazaki, T. Kitawaki, S. Hori, M. Maeda, M. Onodera, T. Uchiyama, S. Fujii, S. Sakaguchi, Crucial role of FOXP3 in the

development and function of human CD4+ CD25+ regulatory T cells, Int. Immunol. 16 (2004) 1643–1656, https://doi.org/10.1093/intimm/dxh165.

[29] S.M. Brunner, G. Schiechl, W. Falk, H.J. Schlitt, E.K. Geissler, S. Fichtner-Feigl, Interleukin-33 prolongs allograft survival during chronic cardiac rejection, Transpl. Int. 24 (2011) 1027–1039, https://doi.org/10.1111/j.1432-2277.2011.01306.x.

[30] M.C. Carroll, V.M. Holers, Innate autoimmunity, Adv. Immunol. 86 (2005) 137–157.

[31] Z. Yang, Y.J. Day, M.C. Toufektsian, Y. Xu, S.I. Ramos, M.A. Marshall, B.A. French, J. Linden, Myocardial infarct–sparing effect of adenosine A2A receptor activation is due to its action on CD4 T lymphocytes, Circulation 114 (2006) 2056–2064, https://doi.org/10.1161/CIRCULATIONAHA.106.649244.

[32] D. Davidson, M. Bakinowski, M.L. Thomas, V. Horejsi, A. Veillette, Phosphorylation-Dependent regulation of T-cell activation by PAG/cbp, a lipid raft-associated transmembrane adaptor, Mol. Cell Biol. 23 (6) (2003) 2017–2028, https://doi.org/10.1128/MCB.23.6.2017–2028.2003.

[33] K. Reginald, K. Chaoui, R. Roncagalli, M. Beau, M.G. Menoita, B. Monsarrat, O. Burlet-Schiltz, M. Malissen, A.G. Peredo, B. Malissen, Revisiting the timing of action of the PAG adaptor using quantitative proteomics analysis of primary T cells, J. Immunol. 195 (2015) 5472–5481, https://doi.org/10.4049/jimmunol.1501300.

[34] V.K. Raker, C. Becker, K. Steinbrink, The cAMP pathway as therapeutic target in autoimmune and inflammatory diseases, Front. Immunol. 7 (2016) 123, https://doi.org/10.3389/fimmu.2016.00123.

[35] K. Molnar-Kimber, L. Yonno, R. Heaslip, B. Weichman, Modulation of TNF alpha and IL-1 beta from endotoxin-stimulated monocytes by selective PDE isozyme inhibitors, Agents Actions 39 (1993) C77–C79.

[36] V.L. Wehbi, K. Taskén, Molecular mechanisms for cAMP-mediated immunoregulation in T cells – role of anchored protein kinase A signaling Units, Front. Immunol. 7 (2016) 222, https://doi.org/10.3389/fimmu.2016.00222.

[37] X. Qi, M. Shao, H. Sun, Y. Shen, D. Meng, W. Huo, Long non-coding RNA SNHG14 promotes microglia activation by regulating miR-145-5p/PLA2G4A in cerebral infarction, Neuroscience 348 (2017) 98–106.

[38] H.K. Eltzschig, O. Eckle, Ischemia and reperfusion—from mechanism to translation, Nat. Med. 17 (2011) 1391–1401.

[39] R.D. Leone, Y.C. Lo, J.D. Powell, A2aR antagonists: next generation checkpoint blockade for cancer immunotherapy, Comput. Struct. Biotechnol. J. 13 (2015) 265–272, https://doi.org/10.1016/j.csbj.2015.03.008.

[40] A. Ohta, R. Kini, A. Ohta, M. Subramanian, M. Madasu, S. Michail, The development and immunosuppressive functions of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway, Front. Immunol. 3 (2012) 1–12, https://doi.org/10.3389/fimmu.2012.00190.

[41] S. Read, V. Malmstrom, F. Powrie, Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation, J. Exp. Med. 192 (2000) 295–302.

[42] C. Oderup, L. Cederbom, C.M. Cilio, F. Ivars, Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4$^+$ CD25$^+$ regulatory T-cell-mediated suppression, Immunology 118 (2006) 240–249, https://doi.org/10.1111/j.1365-2567.2006.02362.x.

[43] S. He, M. Li, X. Ma, J. Lin, D. Li, CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells protect the proinflammatory activation of human Umbilical vein endothelial cells, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 2621–2630, https://doi.org/10.1161/ATVBAHA.110.210492.

[44] V. Bodi, J. Sanchis, J. Nunez, L. Mainar, G. Minana, I. Benet, C. Solano, F.J. Chorro, A. Llacer, Uncontrolled immune response in acute myocardial infarction: Unraveling the thread, Am. Heart J. 156 (2008) 1065–1073, https://doi.org/10.1016/j.ahj.2008.07.008.

[45] S.Z. Josefowicz, L.F. Lu, A.Y. Rudensky, Regulatory T cells: mechanisms of differentiation and function, Annu. Rev. Immunol. 30 (2012) 531–564, https://doi.org/10.1146/annurev.immunol.25.022106.141623.

[46] A. Arce-Sillas, D.D. Álvarez-Luquín, B. Tamaya-Domínguez, S. Gomez-Fuentes, A. Trejo-García, M. Melo-Salas, G. Cárdenas, J. Rodríguez-Ramírez, L. Adalid-Peralta, Review article regulatory T cells: molecular actions on effector cells in immune regulation, J. Immunol. Res (2016) 1–12, https://doi.org/10.1155/2016/1720827.