Acteoside ameliorates diabetic kidney disease via regulating the activation of the PPARγ/β-catenin pathway

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ABSTRACT: Diabetic kidney disease (DKD) is a common complication of diabetes. Acteoside is the main component of Rehmannia glutinosa leaves that shows renal protective effects. This study aimed to explore the protective role of acteoside on podocytes in DKD. Mouse podocyte cell line MPC-5 was stimulated with advanced glycation end products (AGEs) to establish an in vitro DKD model and, then, treated with or without acteoside. The expression levels of miR-27a, PPAR (peroxisome proliferator-activated receptor), and β-catenin were measured at 24 h and 48 h post-treatment. Sprague-Dawley rats were randomly divided into 5 groups: control; DKD; A-1 (DKD + low dose acteoside); A-2 (DKD + medium dose acteoside); and A-3 (DKD + high dose acteoside). The blood and urine biochemical indexes and the renal expressions of miR-27a, PPAR-1γ, and β-catenin were detected. Compared with the control group, the expressions of α-SMA, Snail-1, miR-27a, and β-catenin in AGE-stimulated cells were significantly higher. The effects of acteoside intervention significantly downregulated their expression in AGE-challenged group. The levels of PPARγ and synaptopodin in AGE-stimulated cells were significantly lower than those in the control group, whereas acteoside treatment significantly restored the expressions of these genes. Acteoside alleviated podocyte injury by downregulating miR-27a expression, rescuing the reduction of PPARγ, and inhibiting the activation of β-catenin signaling in the kidney tissues of DKD rats. Acteoside ameliorated podocyte injury in DKD by inhibiting the activation of PPARγ/β-catenin signaling pathway and this process might involve the regulation of miR-27a expression. Our findings suggest a therapeutic potential of acteoside in treating podocyte injury in DKD.

KEYWORDS: acteoside, miR-27a, PPARγ/β-catenin signaling, diabetic nephropathy

INTRODUCTION

Diabetic kidney disease (DKD) is a common complication of diabetes. In recent years, it has become a leading cause of end-stage renal disease in China [1]. Podocyte injury has been identified as a key event in the pathogenesis of DKD, resulting in the disruption of glomerular filtration barrier, podocyte foot process effacement and depletion [2]. The Wnt/β-catenin signaling pathway plays an important role in podocyte damage, and its increased activity has been observed in the podocytes of DKD patients and animal models [3]. The induction of Wnt/β-catenin signaling triggers the transcription of downstream targets, such as α-smooth muscle actin (α-SMA), snail-1, and c-myc [4]. Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the PPAR nuclear receptor family associated with diabetic kidney injury [5]. It has been reported that the activation of PPARγ/β-catenin signaling aggravates podocyte injury in the kidneys of DKD patients [6, 7]. Some studies showed that the expression of PPARγ in obese animal models could be inhibited by the overexpression of microRNA-27a (miR-27a), which promoted the production of inflammatory factors [8, 9]. Also in DKD, PPARγ expression can be increased, and mesangial cell proliferation and fibrosis can be reduced by inhibiting the expression of miR-27a [10].

Acteoside is an extract obtained from Rehmannia glutinosa leaves and has been shown to ameliorate inflammation-induced mesangial cell injury in IgA nephropathy by regulating the chemotaxis and proliferation of Th22 lymphocytes [11]. The renal protective effect of acteoside was also observed in rats with puromycin nephropathy, in which acteoside alleviated puromycin-induced podocyte injury as evidenced by reduced proteinuria, restored
podocyte viability, and suppressed podocyte migration [12]. Thus, we hypothesized that acteoside might exert a renal protective effect on DKD.

In this study, the protective effects of acteoside against DKD injury and the role of PPARγ/β-catenin pathway were investigated using a rat model of streptozotocin (STZ)-induced DKD and an in vitro podocyte injury model.

MATERIALS AND METHODS

AGEs and acteoside

The advanced glycation end products (AGEs) were purchased from Sigma-Aldrich (St. Louis, USA). Acteoside powder was provided by MeiDaKang Pharmaceutical Co., Ltd (Sichuan, China), which contained acteoside (70%) and the structural isoforms of acteoside, including hydroxyacteoside, methoxyacteoside, isoacteoside, and cisacteoside (30%) [13, 14]. The doses of acteoside used in this study were selected as previously described [12].

Cell culture

MPC-5 cells (Catalog no. 5045479161-01) were purchased from the Yuxi Biotechnology Co., Ltd (Jiangyin, China); cultured in DMEM, containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 U/ml of streptomycin, and incubated in a 5% CO₂ incubator at 37°C. The culture medium was replaced every 2–3 weeks. Then, cells were passaged, seeded in 6-well plates and divided into 5 groups: (1) control (normal, remained untreated); (2) AGE-induced podocyte injury model (DKD); (3) DKD + acteoside low dose (A-1, 100 mmol/l); (4) DKD + acteoside medium dose (A-2, 150 mmol/l); and (5) DKD + acteoside high dose (A-3, 200 mmol/l). When cells reached 80% confluence, the culture medium was replaced by serum-free DMEM. AGEs at 200 µg/ml was added into designated groups for 24 h or 48 h. Each group was prepared in triplicate.

Annexin V-FITC/PI apoptosis detection

The adherent cells were digested with EDTA-free trypsin, collected, and centrifuged at 2000 rpm for 5–10 min. Cell pellets were re-suspended in phosphate-buffered saline (PBS, 4°C), washed, and centrifuged at 2000 rpm for 5–10 min. Then, 300 µl binding buffer was added for suspension followed by a 15-min incubation with 5 µl of Annexin V-FITC at room temperature. A volume of 5 µl propidium iodide (PI) was added for staining 5 min before sample loading.

Animal study

Thirty healthy Sprague Dawley rats (150–200 g) were randomly divided into 5 groups (n=5/group): control group (C, remained untreated); DKD group (DKD); DKD + low dose of 10 mg/kg acteoside (A-1); DKD + medium dose of 20 mg/kg acteoside (A-2); and DKD + high dose of 40 mg/kg acteoside (A-3). The STZ administration was performed as previously published [15]. Rats in the DKD group were fed with high fat-diet for 4 weeks, and then intraperitoneally injected with STZ solution (35 mg/kg/day) for 4 days. The blood glucose of each rat was measured bi-weekly since the beginning of high-fat diet intervention. Starting from the first day of STZ injection, blood glucose was monitored every three days. At 72 h after the last injection, blood samples were collected from the tail vein for the detection of fasting blood glucose. Rats with fasting blood glucose of >16.7 mmol/l twice were selected as the diabetic model. Diabetic rats showing >50% increase in 24 h urine volume and 24 h urine protein excretion were considered as successful establishment of DKD rats[16]. Furthermore, the 24-h urine samples in the C and DKD groups were collected to detect urine protein. The acteoside powder was dissolved with normal saline and intraperitoneally injected into rats, once a day, for 5 days. At 2 weeks after the last injection, the 24-h urine of rats was collected with metabolic cages. Then the animals were anesthetized with pentobarbital, sacrificed, and peripheral blood and kidney tissues were harvested.

Blood and urine specimen test

Blood glucose was measured using a Bayer blood glucose meter. The corresponding kits were used to detect blood urea nitrogen (LM-12028, LMAIBio, Shanghai, China), serum creatinine (LM-12068, LMAIBio), random urinary protein, and urinary creatinine (GD-vN9908, Guduo Biotechnology, Shanghai, China). The 24-h urinary protein was detected using an automatic biochemical detector (7170A, Japan).

RT-PCR

A Trizol kit (TransGen Biotech, China; H10318) was used to extract the RNA from collected cells. RNA reverse transcription was performed using a reverse transcriptase kit (Promega), and the reaction system was 20 µl. The primer sequences were shown in Table S1. Reaction conditions: 95°C for 3 min, 95°C for 30 s, and 55°C for 20 s for 40 cycles; then,
72°C for 20 s. Dissolution curve steps: 95°C for 15 s, 60°C for 15 s, warming up for 20 min, and 95°C for 15 s.

The total RNA from kidney tissues was extracted using Trizol RNA extract. RNA reverse transcription was performed using a HiScript II Reverse Transcriptase kit (Vazyme). PCR amplification was performed using a ChamQTM SYBR® qPCR Master Mix fluorescence quantitative kit (Vazyme). The primer sequences were shown in Table S2. Reaction conditions: Stage 1 initial denaturation, 95°C for 30 s; Stage 2 denaturation, 95°C for 10 s; annealing/extension 60°C for 30 s, 40 cycles.

**Western blot**

Cell lysates were prepared and the protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology). The protein (30 μg) was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane by electrophoresis with 15 mA of constant current power. Next, cell protein samples were blocked with 5% skim milk powder for 2 h, and later incubated with the following primary antibodies: PPARγ (1:1000 dilution, Ab-mart, Berkeley Heights, USA) and β-catenin (1:500 dilution, HuaGene, Shanghai, China) at 4°C overnight. Then, the membrane was washed with TBST, added with a secondary antibody (1:2000), and incubated for 2 h. The membrane was colored, exposed, and scanned to obtain the films. With β-actin (Catalog no. 1854-s, HuaAn, Hangzhou, China) as the internal control, the expressions of PPARγ and β-catenin were detected.

The kidney tissue specimens were levigated and homogenized, and the tissue protein was extracted using phenylmethanesulfonyl fluoride/PMSF solution. The protein concentration was determined by BCA, and 30 μg were separated by gel electrophoresis. The protein was, then, transferred onto a membrane, blocked with 5% dried skimmed milk, and incubated with the following primary antibodies: PPARγ (1:1000 dilution, Ab-mart, Berkeley Heights, USA) and β-catenin (1:500 dilution, HuaGene, Shanghai, China) at 4°C overnight. Then, the membrane was washed with TBST, added with a secondary antibody (1:2000), and incubated for 2 h. The membrane was colored, exposed, and scanned to obtain the films. With β-actin (Catalog no. 1854-s, HuaAn, Hangzhou, China) as the internal control, the expressions of PPARγ and β-catenin were detected.

**Immunohistochemistry**

Cells and kidney tissues were fixed with 2.5% glutaraldehyde phosphate buffer, embedded in epoxy resin, and sectioned. Immunohistochemical staining was used to assess the expression of α-SMA, Snail-1, synaptopodin, β-catenin, or PPAR-γ (1:100 dilution, Maxim, Fuzhou, China). Positive staining was defined as brown or brownish-yellow stains in the cytoplasm under light microscope. An automatic digital imaging system was used for imaging. Then, the expression situations were analyzed using ImageJ software, and 10 discontinuous visual fields were evaluated.

**Statistical analysis**

All samples were analyzed in triplicate. The cell culture experiments were repeated three times. Data were analyzed using SPSS 17.0. Normal distributions of quantitative data were expressed as mean ± standard deviation. Repeated measurement data were analyzed using repeated measures analysis of variance (ANOVA). Comparison among groups was conducted using one-way ANOVA. p < 0.05 was considered statistically significant; * indicates significant difference when compared with the control group at the same time point, # indicates significant difference when compared with the DKD group at the same time point, ** p < 0.01, ## p < 0.01; # indicates significant difference when compared with the control group at the same time point, * p < 0.05, ## p < 0.01.

**RESULTS**

**Acteoside suppressed apoptosis and regulated the RNA expressions of miR-27a, PPAR-γ, and β-catenin in AGE-induced MPC-5 cells**

Cells treated with AGEs, when compared with the control group, showed a significantly higher apoptosis rate at both 24 h and 48 h post-treatments (p < 0.01). However, the intervention of acteoside in A-1, A-2, and A-3 significantly suppressed AGE-stimulated apoptosis in MPC-5 podocytes (p < 0.01, Fig. 1A). Compared with the control, the expression of miR-27a in MPC-5 cells was significantly increased by AGE stimulation at both 24 h and 48 h after acteoside treatment (p < 0.05). The treatment of acteoside at all doses significantly decreased the miR-27a level at both time points (p < 0.05). In A-3 group, the level of miR-27a was similar to that in the control group (p > 0.05) (Fig. 1B). AGE-treated
Fig. 1 Effects of acteoside on the apoptosis rates and the expressions of miR-27a, PPARγ and β-catenin in AGE-induced MPC-5 cells. MPC-5 cells received designated doses of acteoside for 24 or 48 h followed by the detection of (A) apoptosis rate and the measurements of (B) miR-27a, (C) PPARγ, and (D) β-catenin expressions.

Fig. 2 Effects of acteoside on the protein expressions of PPARγ and β-catenin in AGE-treated MPC-5 cells. The expressions of PPARγ and β-catenin in MPC-5 cells were measured using Western blot at 24 h or 48 h post-treatment.
cells showed significantly reduced expression of PPARγ as compared with the control group (p < 0.05), whereas acteoside intervention successfully restored PPARγ expression at all doses (p < 0.05) (Fig. 1C). The robust effect of acteoside was observed when the high concentration (200 mmol/l) was applied. The level of β-catenin was significantly induced by AGE treatment (p < 0.05) but suppressed by acteoside at all doses (p < 0.05). The levels of β-catenin between the A-3 group and the control showed no statistical significance (p > 0.05, Fig. 1D). Consistently, AGE stimulation inhibited the protein expression of PPARγ and induced the level of β-catenin in MPC-5 cells in comparison with the control (p < 0.05). The intervention of acteoside at all doses effectively promoted the levels of PPARγ and downregulated the β-catenin expression in injured MPC-5 cells (p < 0.05, Fig. 2).

Effects of acteoside on the protein expression of α-SMA, Snail-1, and synaptopodin in AGE-treated podocytes

Next, we performed immunohistochemistry in cells at 24 h and 48 h after acteoside treatment. The levels of α-SMA and Snail-1 in DKD group were significantly higher than those in the control (p < 0.05). The treatment of acteoside at all concentrations effectively downregulated AGE-induced expressions of α-SMA and Snail-1 (p < 0.05). The synaptopodin level in injured podocytes, however, was significantly lower than that in the control group (p < 0.05). Acteoside at all doses (A-1, A-2, and A-3) significantly upregulated synaptopodin expression in AGE-treated cells (p < 0.05, Table 1).

Effects of acteoside on blood and urine biochemical indicators in diabetic rats

To explore the effects of acteoside on DKD in vivo, an animal model of STZ-induced diabetes was established. When compared with the control group (p < 0.05), diabetic rats showed significantly higher levels of blood glucose, urea nitrogen, serum creatinine, 24-h urinary protein, and the urinary protein-to-creatinine ratio. Acteoside at medium and high doses (A-2 and A-3) efficiently decreased all blood and urine biochemical indicators in diabetic rats (p < 0.05, Table 2).

Effects of acteoside on the expressions of α-SMA, Snail-1, synaptopodin, miR-27a, PPAR-γ, and β-catenin in rats with diabetic kidney injury

DKD group showed significant increased levels of α-SMA, Snail-1, miR-27a, and β-catenin; and decreased expressions of synaptopodin and PPAR-γ when compared with the control (p < 0.05). In the A-2 and A-3 groups, the renal RNA expressions of α-SMA, Snail-1, miR-27a, and β-catenin were effectively downregulated; and the levels of synaptopodin and PPAR-γ were greatly increased (p < 0.05, Fig. 3). When compared with the control, rats with diabetes showed significantly higher protein expressions of α-SMA, Snail-1, and β-catenin, whereas the levels of synaptopodin and PPAR-γ were significantly decreased. The treatment of high dose acteoside (200 mmol/l) showed the robust effect on suppressing the induction of α-SMA, Snail-1, and β-catenin; and restoring the levels of synaptopodin and PPAR-γ (p < 0.05, Fig. 4). Consistently, immunohistochemistry data showed that the renal expressions of α-SMA, Snail-1, and β-catenin in the DKD group were higher than those in the control. However, the expressions of PPARγ and synaptopodin were lower in DKD when compared with the control. The intervention of acteoside effectively reversed diabetes-induced aberrant expressions of these proteins (Fig. 5).

DISCUSSION

Acteoside is the main compound of *Rehmannia glutinosa*. It belongs to phenylethanoid glycosides whose effects are known for nourishing the Yin, tonifying the kidney, and improving intestinal flora imbalance in diabetic nephropathy animals [14, 17, 18]. Previous studies revealed that the expression of calcineurin is significantly increased in kidney tissues of STZ-induced early diabetic animal models [19, 20], while another study demonstrated that acteoside had the effect of inhibiting the expression of calcineurin in the kidney of diabetic nephropathy animals [21]. This indirectly suggests that acteoside may be a potential effective drug for DKD treatment.

MiRNA is an endogenous single-stranded small RNA molecule, which participates in the regulation of gene expression, and influences various biological processes [22–24]. The plasma level of miR-27a can reflect the degree of renal tubulointerstitial fibrosis in DKD, and the mechanism is that miR-27a inhibits PPARγ expression and activates the TGF-β/Smad signaling pathway [25–27]. A study also confirmed that in DKD, β-catenin activation was one of the mechanisms that mediated podocyte injury [28]. Therefore, miR-27a, PPARγ and β-catenin all play important roles in the pathogenesis of DKD. Meizhou Zhan et al [6] confirmed that miR-27a and activation of the PPARγ/β-catenin pathway were mechanisms leading to the
Fig. 3 Effects of acteoside on the RNA expressions of α-SMA, Snail-1, synaptopodin, miR-27a, PPAR-γ, and β-catenin in rats with STZ-induced DKD. Rats received designated doses of acteoside and their kidney tissues were collected. The expressions of α-SMA, Snail-1, synaptopodin, miR-27a, PPAR-γ, and β-catenin were measured using RT-PCR.

Fig. 4 Effects of acteoside on the protein expressions of α-SMA, Snail-1, PPAR-γ, β-catenin, and synaptopodin in DKD rats. Rat kidney tissues were harvested after the treatment; and the protein expressions of α-SMA, Snail-1, PPAR-γ, β-catenin, and synaptopodin were assessed using Western blot.
aggravation of podocyte injury in DKD. Thus, miR-27a may be a new potential for DKD treatment.

In our study, AGEs were used to induce podocyte injury modeling. The apoptosis rate of podocytes in AGE-treated cells was significantly higher than that in the control group, while acteoside intervention significantly reduced the apoptosis of AGEs-injured cells at 24 h and 48 h after treatment. Compared to the control group, the expression levels of miR-27a, PPARγ and β-catenin in the DKD group were significantly increased. After the incubation with acteoside for 24 h and 48 h, the expressions of miR-27a and β-catenin were greatly decreased and the level of PPARγ was restored in AGE-treated podocytes. Further immunohistochemical staining showed that acteoside significantly decreased the expressions of α-SMA and Snail-1, but elevated the level of synaptopodin in MPC-5 cells. α-SMA and snail-1 are the two downstream targets of the Wnt/β-catenin signaling pathway [29]. Synaptopodin has been identified as a classical podocyte-specific marker [30]. Our findings suggested that acteoside alleviated AGE-induced podocyte injury via suppressing the expression of miR-27a and the activation of Wnt/β-catenin signaling pathway. We further explored the therapeutic effect of acteoside on DKD in vivo. The levels of proteinuria and serum creatinine in diabetic rats were alleviated by acteoside at different doses. Consistent with the in vitro data, the treatment of acteoside successfully reversed diabetes-induced aberrant expressions of miR-27a, α-SMA, Snail-1, β-catenin, PPARγ, and synaptopodin in rats with STZ-induced diabetes.

The most robust therapeutic effect was observed in the A-3 group. There were limitations in the present study. First, the effect of acteoside on the levels of blood pressure, blood lipids, and HbA1c were not explored. Also, the overexpression, or silencing, of miR-27a was not further verified; and the indicators were not detected at more time points in the animal experiments. Further investigations are needed to elucidate the molecular mechanisms of acteoside on the protective effect in DKD.

**CONCLUSION**

Podocyte injury plays an important role in DKD development. Our results demonstrated that acteoside intervention alleviated podocyte injury both in vitro and in vivo, by decreasing podocyte apoptosis, downregulating miR-27a expression, rescuing the reduction of PPARγ, and inhibiting the activation of β-catenin signaling. These results suggested a therapeutic potential of acteoside in alleviating podocyte injury in DKD, subsequently reducing proteinuria and protecting kidney function.
Fig. 5 Immunohistochemical staining of rat kidney tissues. Sectioned rat kidney tissues were stained with α-SMA, Snail-1, synaptopodin, PPAR-γ, and β-catenin using immunohistochemistry.

Appendix A. Supplementary data
Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.034.

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Appendix A. Supplementary data

Table S1 The primer sequences for RT-PCR of the RNA from collected cells.

| Primer       | Sequence                              |
|--------------|---------------------------------------|
| GAPDH-F      | ACAGCAACAGGGGTGGTGGAC                 |
| GAPDH-R      | TTTGAGGGTGACGGCAAACCTT                |
| PPARγ-F      | TGATATCGACCAGCTGAA-CC                 |
| PPARγ-R      | ATGGCCACCTCT-TTGCTCT                  |
| β-catenin-F  | ATGTGGTCACCTG-TGCAGCT                 |
| β-catenin-R  | GGCTCAGTGATGATCTCTCCCT                |
| miR-27a      | GGCATT-ACAGTGCTAAGT                   |

Table S2 The primer sequences for RT-PCR of the total RNA from kidney tissues.

| Primer       | Sequence                              |
|--------------|---------------------------------------|
| GAPDH-F      | ACTGAGAGCAAGAGAGAGGC                  |
| GAPDH-R      | TTGTATGGGCTCTGGGATGG                  |
| Snail 1-F    | AGCCCA-ACTAGCGAGCTG                   |
| Snail-1-R    | CCAAGGAGGTTCCAGATG                   |
| PPARγ-F      | CCCTGCAAAGCA-TTTGTAT                  |
| PPARγ-R      | ACTGGCACCCTGAAAAATG                   |
| miR-27a      | AGGG-CTTAGCTGCTTGTGAGCA               |
| synaptopodin-F| CACCACAGCAGCTCAACA                   |
| synaptopodin-R| TGCTG-GACCTCACTTCTCTCT               |
| β-catenin-F  | GCTTC-TGTTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC