A dual intervention of triiodothyronine and baicalein bidirectionally upregulates Klotho with attenuation of chronic kidney disease and its complications in aged BALB/c mice

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Abstract

Chronic kidney disease (CKD) presents a pressing global health challenge characterized by progressive renal function decline and increased morbidity and mortality. The interplay between CKD and hypothyroidism, particularly through nonthyroidal low-triiodothyronine (T3) syndrome, exacerbates disease progression and increases mortality rates. Perturbations in the hypothalamic–pituitary–thyroidal (HPT) axis contribute to this scenario, whereas fibrotic kidneys exhibit diminished levels of the protective protein Klotho owing to abnormal activation of the Wnt–β-catenin pathway. Leveraging our previous findings showing the ability of T3 to downregulate aberrant Wnt/β-catenin pathway activity by increasing Klotho expression, both in vitro and in vivo, and Baicalein's direct inhibition of the Wnt pathway in *C. elegans*, we investigated the potential of Klotho as a molecular link between CKD and hypothyroidism. Through experiments utilizing an adenine-induced CKD aged mouse model, we aimed to increase Klotho expression via exogenous T3 administration and bidirectional Wnt pathway blockade via baicalein (BAI). Our results demonstrated a significant synergistic upregulation of Klotho expression with combined T3 and BAI treatment, surpassing the effects of individual treatments. Moreover, this combination therapy effectively suppressed aberrant signaling molecules such as transforming growth factor beta (TGF), nuclear factor kappa B (NFκB), and glycogen synthase kinase 3 (GSK3), thus mitigating renal fibrosis. Improvements were observed in CKD-induced complications, including cardiovascular disorders, dyslipidemia, and alterations in bone and serum markers. This unique bidirectional approach, in which Klotho biology is directly enhanced by T3 and sustained by T3 coupled with Wnt pathway blockade via BAI, presents a promising strategy for CKD management. This approach has the potential to ameliorate renal degradation, which is particularly relevant for elderly CKD patients with hypothyroidism. Our findings underscore the substantial therapeutic potential of hormone and natural chemical interventions in CKD management.

Keywords: Chronic kidney disease, Triiodothyronine, Klotho, Wnt/β-catenin, Aging, Baicalein

Abbreviation

T3, Triiodothyronine; BAI, Baicalein; M-Kl, Membrane Klotho; S-Kl, Soluble Klotho; Sp-Kl, Spliced Klotho; α-Kl, Alpha Klotho; Wnt, Wingless-related integration site; AI, Atherogenic index; BUN, Blood urea nitrogen; CKD, Chronic kidney disease; CVD, Cardiovascular disease; GSK-3β, Glycogen synthase kinase 3 beta; TRPC6, Transient receptor potential cation channel; TRPV5, Transient receptor potential cation channel subfamily V member 5; TGF-β,

Transforming growth factor-beta; TC, Total cholesterol; TG, Triglyceride; LDL, Low-density lipoprotein; V-LDL, Very low-density lipoprotein.

Introduction

The prevalence of chronic kidney disease (CKD) has accelerated worldwide and is becoming a severe health problem [1]. Recent studies suggest that an abnormal increase in CKD, especially in developing Asian countries, is associated with hypertension, type 2 diabetes, and cardiovascular disease (CVD) [2]. The increasing CKD trend can be correlated with the aging population in the last several decades. Consistent with this concept, many studies suggest that aging is an independent risk factor for the development and progression of CKD [3,4]. Many reports also indicate that CKD is associated with numerous complications, including dyslipidemia, CVD, mineral bone disorder, and thyroid dysfunction [5]. The kidney plays an essential role in thyroid hormone metabolism, degradation, and excretion [6]. Reduced expression of both triiodothyronine (T3) and thyroxine (T4) with elevated levels of thyroid-stimulating hormone (TSH) is commonly observed in CKD patients because of dysregulation of the hypothalamus–pituitary–thyroid (HPT) axis [7]. Additionally, a reduction in the glomerular filtration rate (GFR) impairs the conversion of T4 to T3 through uremica. This may lead to increased mortality in uremic patients [8]. The suppression of T3 in uremica may involve chronic inflammation, malnutrition, and vascular injury [9].

Klotho is a single-pass transmembrane protein detectable in limited tissues, particularly in distal convoluted tubules of the kidneys and the choroid plexus in the brain. The Klotho protein may regulate the occurrence of age-related disorders or natural aging processes by functioning as a circulating humoral factor [10]. The Klotho protein is approximately 130 kDa (1,014 amino acids) with a presumed signal sequence at the N-terminus, a single transmembrane domain at the C-terminus, and a short cytoplasmic domain (10 amino acids) [11,12]. Membrane Klotho is proteolytically cleaved and shed by different types of secretases, such as α, β, and γ secretases [13–15]. There is also a small secreted form of Klotho produced by alternative splicing consisting of 550 amino acids, a truncated Klotho protein [16]. Clinical studies have suggested that both the membrane and secreted forms of Klotho are downregulated in human CKD, demonstrating that the loss of Klotho is correlated with the pathogenesis of kidney injury [17,18].

Building upon this foundation, recent investigations have sought to elucidate the mechanistic link between CKD and hypothyroidism through the lens of Klotho biology. Notably, our prior studies revealed that T3, a thyroid hormone, could increase Klotho expression in *Caenorhabditis elegans* (*C. elegans*).[19] This raises intriguing questions about the potential

benefits of T3 supplementation in mitigating the impaired HPT axis and conferring protective effects against age-associated CKD, particularly in higher mammalian models.

Moreover, the role of the evolutionarily conserved Wnt/β-catenin pathway in the pathogenesis of CKD is well established. Recent observations suggest that aberrant activation of the Wnt/β-catenin pathway occurs in many types of kidney disease, including CKD [20,21]. Wnt proteins secrete extracellular signal molecules that play essential roles in the formation of the nephron and renal development [22]. Wnt proteins interact with their receptors and coreceptors and induce downstream signaling events, which leads to the stabilization of β-catenin. Excess stabilized β-catenin accumulation controls downstream protein expression, leading to matrix production and fibrosis [23–25]. Overactivation of the Wnt pathway in kidney diseases also inhibits Klotho expression [26]. In contrast, Klotho can protect against CKD by inhibiting the Wnt pathway [27–29]. Furthermore, in our prior study involving the nematode *C. elegans*, we discovered a significant association: suppression of the Wnt pathway leads to increased Klotho expression [30]. Building on these observations, our previous research revealed that T3 can notably augment various forms of kidney-specific Klotho protein while reducing the increase in Wnt/β-catenin pathway activity in aged mice [31]. This reciprocal regulatory mechanism prompts inquiries into its evolutionary conservation across species and potential implications for chronic kidney disease.

Baicalein (BAI) is a component of the root of *Scutellaria baicalensis* and possesses many beneficial properties, including anti-inflammatory, antioxidative, antimicrobial, and immunoregulatory effects. It has a time-honored place in Asian traditional medicine for treating liver, kidney, and cardiovascular diseases [32]. BAI has been reported to protect against different types of carcinoma and nephrotoxicity by inhibiting the Wnt/β-catenin pathway [33–35]. Preliminary studies from our laboratory using the nematode *C. elegans* revealed that BAI could increase Klotho expression by inhibiting Wnt ligands [30].

This study proposes a novel bidirectional targeting approach that leverages both exogenous hormone (T3) and the natural compound BAI to achieve stable upregulation of Klotho. The rationale behind this dual intervention is grounded in the potential synergistic effects of hormonal and natural compound interventions, aiming for enhanced protective effects against CKD. This integrative strategy holds promise for advancing our understanding of CKD pathophysiology and developing innovative therapeutic avenues.

As we delve into this intricate landscape of CKD, the multifaceted interplay between thyroid function, Klotho biology, and the Wnt/β-catenin pathway requires further exploration. By

unraveling these complexities, we strive not only to expand our scientific knowledge but also to pave the way for targeted and effective interventions that can alleviate the burden of CKD on a global scale.

Materials and methods

Animal care and treatment

Animal studies were performed after approval by the Institutional Animal Ethics Committee (IAEC) of Pondicherry University, India (IAEC approval ref. No: PU/CAHF/23rd IAEC/2019/03, dated 15.04.2019). All the experiments were conducted in accordance with the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, under the supervision of the IAEC. The animals were housed under conventional conditions: $23^0 \pm 2^0$ C, 12:12 light:dark cycle, standard diet, and water *ad libitum*. Twenty-month-old BALB/c mice were used for this study. After 15 days of acclimatization, the animals were grouped, with six animals in each group, on the basis of their weight. Blood collection and euthanasia of the animals were carried out under isoflurane narcosis. All experimental animals were approved in advance by the Institutional Animal Ethics Committee (IAEC). CKD was induced by the addition of 50 mg/kg b.w. adenine (Sigma Aldrich, USA) dissolved in 0.5% carboxy methyl cellulose (CMC) via an oral dose for 21 days, whereas the control animals received 0.5% CMC for only 21 days. After CKD induction, the animals were further divided into different groups. The CKD group received a vehicle for T3 and BAI (0.1 N NaOH and DMSO) and was named the placebo group. The other groups received an individual dose of either 1 mg/kg b.w. T3 or 75 mg/kg b.w. Baicalein (BAI) (Sigma Aldrich, USA) was administered after treatment. The T3 and BAI doses were fixed on the basis of previous reports [36–40]. However, the supraphysiologic dose of T3 has been chosen to monitor its physiological effect on Klotho protein expression in the kidney. The last group received the combined treatment (T3 + BAI group). BAI was administered for five consecutive days after 21 days, whereas T3 was administered during the last three days, i.e., the 24th–26th days. All the animal experiments used in the manuscript followed the recommendations of the ARRIVE guidelines. The experimental design is outlined in **Fig. 1.**

Serum and urine biochemical parameters

Serum creatinine, urea, blood urea nitrogen (BUN), albumin, calcium, inorganic phosphorus, hemoglobin, total cholesterol (TC), total triglyceride (TG), high-density lipoprotein (HDL-C), alkaline phosphatase (ALP) activity, bilirubin, urine creatinine, and urea were measured via the

Agappe diagnostic kit (Agappe Diagnostic, India) according to the manufacturer's instructions. LDL-C and VLDL-C were calculated via the equation provided by Sampson et al. [41] as follows:

$$\text{LDL-C} = \frac{TC}{0.948} - \frac{\text{HDL-C}}{0.971} - \left(\frac{TG}{8.56} - \frac{TG \times \text{Non-HDL-C}}{2140} - \frac{TG^2}{16100} - 9.44\right)$$

$$\text{VLDL-C} = \frac{TG}{8.59} + \frac{TG \times \text{Non-HDL-C}}{2250} - \frac{TG^2}{16500}$$

ELISA

T3 (E-EL-0079), TSH (E-EL-M1153), and 1,25 hydroxy Vit-D3 (E-EL-0016) ELISA kits were purchased from Elabscience USA. T3 and Vit-D3 levels were measured via competitive ELISA, and TSH levels were measured via the sandwich ELISA method according to the manufacturer's instructions.

Histological analysis

Kidney tissue was harvested and fixed in 10% neutral buffered formalin, and a 4-µm-thick section was prepared from paraffin-embedded tissue with the help of an expert histopathologist. The kidney sections were further stained with H&E and Masson's trichrome. Kidney damage was evaluated on the basis of parameters such as inflammatory infiltration, protein casts, interstitial fibrosis, and ECM deposition. The parameters were measured in ten randomly selected fields from each section at 400x magnification. All the parameters were measured via ImageJ software.

RNA isolation and qRT–PCR analysis

RNA express reagent (Hi-Media, India) was used to isolate total RNA from mouse kidney tissue. Briefly, 2 µg of RNA was subjected to cDNA synthesis via a cDNA isolation kit (Thermo Fisher Scientific, USA) per the manufacturer's instructions. cDNA was subjected to qPCR using SYBR green master mix (Roche, USA). The qPCR was run on a Hi-Media Insta Q48™ real-time machine for 40 cycles. mRNA was quantified via the $2^{-\Delta\Delta Ct}$ method, with β-actin used as an internal control [42]. The details of the primers used are listed in **Table 1**.

Western blotting analysis

For western blot analysis, all reagents were purchased from Sigma Aldrich, USA, unless otherwise indicated. Kidney tissue was homogenized in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Equal concentrations of proteins were loaded and electrophoresed on a 10% gradient tris-glycine gel. Proteins were electrophoretically

transferred to an immobile-P PVDF membrane (Roche, USA). After being blocked with 5% nonfat skim milk for 1 h at room temperature, the membranes were incubated with primary antibodies (Santacruz, USA) against anti-Klotho (sc-515942), anti-GSK3β (sc-7291), anti-β-catenin (sc-7963), anti-CK1 (sc-74582), anti-NFκB (sc-8008), anti-IL6 (sc-57315) and anti-GAPDH (sc-32233) at 4°C overnight. After thorough washing with buffers, the membranes were incubated with a secondary antibody (sc-516102) for 1 h at room temperature. The bands were then developed in a chemiluminescence substrate via the Vilber Chemi Doc imaging system. The intensity of each band was measured via ImageJ software, and the relative expression was determined with respect to that of GAPDH.

Antioxidant enzyme assay

Catalase assay

Catalase activity was measured as described previously [43]. The tissue homogenate was incubated in the substrate at 37°C for three minutes. After incubation, the reaction was stopped with ammonium molybdate. The absorbance of the molybdate and hydrogen peroxide yellow complex was measured at 374 nm against a blank. The specific activity was expressed as U/mg of protein.

SOD activity

SOD activity in the tissue homogenate was measured via the methods of Kostyuk V A et al. [44]. SOD activity was measured by the inhibition of autooxidation of quercetin by the enzyme at pH 10. The results are expressed as U/mg of protein.

Statistical analysis

Two-way analysis of variance with post hoc Tukey correction was used to compare the means of different groups. All experiments were performed in triplicate, and the results are reported as the means ± SEMs. The graphs were plotted via GraphPad Prism software (GraphPad Software, San Diego, CA, USA), and statistical significance was also measured via the same software. The error bars represent the SEM. p values < 0.05 were considered statistically significant.

Results:

CKD reduces body weight and improves with bidirectional treatment

The body weights of all the animals were measured every two days during the treatment period. The body weights of the animals significantly decreased during the adenine administration

period. At the adenine diet endpoint (21st day), the adenine-fed placebo group animals weighed 31% less than the control group did ($p<0.001$). In contrast, when adenine-fed animals were treated with T3, BAI, or the combined dose (T3 + BAI) alone, significant improvements in body weight at the end of the treatments (27th day) were observed. The T3-treated animals presented an increase in body weight of 17.9% ($p<0.001$), the BAI-treated animals presented an increase of 16.6% ($p<0.001$), and the combined (T3 + BAI)-treated animals presented the greatest increase of 19.2% ($p<0.001$) from the 27th-day placebo group body weight **(Fig. 2).**

Bidirectional treatment ameliorates abnormal serum and urine parameters in CKD animals

The present study of adenine-induced CKD in aged animals assessed kidney injury via serum and biochemical kidney injury markers. We initially observed that the serum creatinine, urea, and BUN concentrations were significantly greater in the placebo group than in the control group. The percentage of increase in creatinine was 8.8-fold ($p<0.001$), that in urea was 1.56-fold ($p<0.05$), and that in BUN was 1.56-fold ($p<0.05$). In contrast, the urine creatinine and urea concentrations in the placebo group were significantly lower than those in the control group (3.12-fold ($p<0.01$) and 2.08-fold ($p<0.01$), respectively). Moreover, the urine ALB concentration increased 3.05-fold ($p<0.001$) in the placebo group than in the control group, leading to significant albuminuria. However, treatment with either T3, BAI or their combination (T3 + BAI) ameliorated the changes observed in the placebo group toward the normal range. Specifically, in comparison with the treated groups, the combined (T3 + BAI) treatment produced a more pronounced effect, in which the serum creatinine, urea and BUN levels were 3.8-fold ($p<0.01$), 1.63-fold, ($p<0.01$), and 1.68-fold ($p<0.001$) lower, respectively, than those in the placebo group. **(Table 2)**

Effects of T3, BAI, and the combined treatment (T3 + BAI) on kidney fibrosis and extracellular matrix deposition in CKD animals

Kidney sections were subjected to H&E and Masson's trichrome staining to assess pathological renal conditions **(Fig. 3).** H&E staining **(Fig. 3a)** revealed that CKD mice in the placebo group presented significant monocyte and lymphocyte infiltration (A), protein casts (B), and cytoplasmic vacuolation (C) in renal sections**.** Moreover, the Masson's trichrome-stained **(Fig. 3b)** renal sections indicate an increase in interstitial fibrosis with a high deposition of extracellular matrix components in the placebo group. However, treatment with T3, BAI or their combination (T3 + BAI) significantly improved the damage. The damage to the renal sections was evaluated on the basis of ten randomly selected fields per section, which were

examined at 400x magnification. The evaluation criteria and observed results are presented in **Table 3**. Overall, our results showed that the combined treatment produced a better counteractive effect on renal fibrosis and ECM accumulation.

Combined treatment (T3 + BAI) maximally counteracts the decline in kidney Klotho levels during CKD

Recent evidence has shown that Klotho can serve not only as an early biomarker of CKD but also as a potential therapeutic target for CKD [45,46]. In our study of adenine-induced CKD, we found that the mRNA expression of M-Klotho and Sp-Klotho was significantly lower in the placebo group than in the control group by 3.25-fold ($p<0.001$) and 2.35-fold ($p<0.001$), respectively **(Fig. 4a, c).** In contrast, M-Klotho expression increased significantly in the T3, BAI, and combined (T3 + BAI) groups by 4.5-fold ($p<0.001$), 3.6-fold ($p<0.01$), and 5.5-fold ($p<0.001$), respectively, compared with that in the placebo group **(Fig. 4b).** Similarly, Sp-Klotho expression also increased significantly upon T3, BAI, and combined (T3 + BAI) treatment by 4.2-fold ($p<0.001$), 2.4-fold ($p<0.05$), and 5.3-fold ($p<0.001$), respectively **(Fig. 4d).** The protein expression of Klotho revealed that α-KL and SKL expression was markedly lower in the placebo-treated group than in the control group (91.6% and 15.5%, respectively) **(Fig. 4e, f, g)**. Upon treatment with T3 and BAI, a significant increase in Klotho protein expression was observed, and the combined treatment (T3 + BAI) resulted in greater Klotho protein expression than either of the individual treatments did.

Aberrant increase in GSK-3β expression during CKD is maximally suppressed by T3 + BAI

Recent studies have also implicated GSK3β in diverse kidney diseases, and targeting GSK3β improved kidney fibrosis [47,48]. To address this issue, we evaluated the expression of GSK-3β, which tends to be overexpressed in different types of kidney injury. We found a significant 3.3-fold ($p<0.001$) increase in GSK-3β protein expression in the kidneys of the adenine-fed placebo group compared with the control group. Interestingly, compared with the placebo, the T3 and BAI treatments counteracted the observed changes in expression by 2.1-fold ($p<0.001$) and 1.8-fold ($p<0.01$), respectively. The optimal downregulation of GSK3β, with a decrease of 4.6-fold ($p<0.001$), was observed in the combined (T3 + BAI)-treated group **(Fig. 5a, b)**.

Combined treatment (T3 + BAI) suppresses the canonical Wnt/β-catenin pathway in CKD animals

Understanding the crosstalk of the evolutionarily conserved Wnt/β-catenin pathway with other signaling pathways is crucial for understanding kidney disease progression and elucidating the therapeutic potential of treatment protocols [49]. To determine the effects of our treatments on the canonical Wnt/β-catenin pathway, the mRNA expression of Wnt ligands such as Wnt1, 3, 8A, 8B, and 10A and the protein expression of β-catenin and casein kinase 1 (CK-1) were measured. We found that the mRNA expression of all Wnt ligands increased significantly in the placebo-treated mice compared with the control mice **(Fig. 6a).** However, treatment with BAI alone or in combination (T3 + BAI) repressed the expression of these mRNAs compared with that in the placebo group **(Fig. 6b).** In contrast, no significant ($p<0.99$) changes were observed in the group that received only the T3 treatment.

At the level of β-catenin protein expression, we observed a significant increase of 97.9% ($p<0.001$), and CK-1 expression was 38.5% ($p<0.01$) lower in the placebo group than in the control group. In contrast, after the T3 and BAI treatments, the protein expression of β-catenin decreased significantly [T3: 27.6% ($p<0.01$); BAI: 17.8% ($p<0.01$)], and CK-1 [T3: 58.5% ($p<0.01$); BAI: 50.4% ($p<0.01$)] increased significantly toward a normal level. Moreover, the combined treatment group exhibited a maximum decrease in β-catenin [58.3% ($p<0.01$)] with increasing CK-1 [71% ($p<0.01$)] protein expression compared with the placebo group. **(Fig. 6c, d, e).**

Treatments can modulate kidney TGF-β expression

Regarding the genesis of CKD, the role of TGF-β as an essential mediator of the whole process has been recognized recently [50]. In the present study, we found that the expression of TGF-β mRNA increased significantly, by 4.1-fold ($p<0.001$), in the placebo group compared with the control group **(Fig. 7a).** Upon treatment with either T3 and/or BAI, a significant reduction in TGF-β mRNA expression of 2.3-fold ($p<0.05$) and 2.6-fold ($p<0.05$) was observed compared with that in the placebo group. In comparison, the combined (T3 + BAI) group presented a 3.56-fold greater reduction in TGF-β mRNA expression ($p<0.05$) than the placebo group did **(Fig. 7b).**

Protective effect against CKD-induced inflammation

A proinflammatory state is also linked to chronic kidney disease, and persistent low-grade inflammation is increasingly recognized as a defining feature of CKD [51,52]. Increased production and decreased clearance of proinflammatory cytokines contribute to the chronic inflammatory status in CKD [53,54]. Thus, we analyzed the expression of cytokines and

inflammatory immune cell accumulation in the kidneys to assess the intensity of renal inflammation.

The protein expression levels of inflammatory kidney markers, such as nuclear factor-κB (NF-κB) and interleukin 6 (IL-6), were significantly greater in the placebo-treated mice than in the control mice. This includes increases in NF-κB by 2.7-fold ($p \leq 0.001$) and in IL-6 by 3-fold ($p \leq 0.001$). Moreover, the T3 and BAI groups presented significantly lower levels of inflammatory markers than did the placebo group. Moreover, compared with the placebo treatment, the individual T3 and BAI treatments significantly reduced N-FκB expression by 2.2-fold ($p \leq 0.01$) and 1.8-fold ($p \leq 0.01$), respectively. Similarly, compared with the placebo, T3 and BAI significantly reduced IL-6 expression by 1.5-fold ($p \leq 0.01$) and 1.65-fold ($p \leq 0.01$), respectively. The maximum reduction in inflammatory markers was observed in the combined treatment group, in which NF-κB expression was reduced by 4.3-fold ($p \leq 0.001$) and IL-6 expression was reduced by 3-fold ($p \leq 0.001$) compared with those in the placebo group **(Fig. 8a, b, c).** These findings provide evidence that combined T3 + BAI could alter the increase in inflammatory cytokines during CKD, thereby protecting the kidneys from further deterioration.

T3 but not BAI could protect CKD animals from anemia

A decrease in one or more important red blood cell parameters, such as the hematocrit, hemoglobin concentration, or red blood cell count, is referred to as anemia. Since anemia is a frequent side effect of CKD, it is unquestionably an independent risk factor for worse outcomes in CKD patients. Anemia is less common in early kidney disease, and it often worsens as kidney disease progresses and when more kidney function is lost [55,56]. Hence, we were interested in determining the anemia status in CKD mice and the effect of treatment on anemia status. The hematocrit percentage and hemoglobin concentration were significantly lower in the placebo group than in the control group by 27.4% ($p \leq 0.05$) and 23.3% ($p \leq 0.05$), respectively. Compared with those in the placebo group, the hematocrit percentages in the T3- and combined (T3 + BAI)-treated groups were 17.08% ($p \leq 0.05$) and 15.7% ($p \leq 0.05$), respectively. Similarly, the Hb concentration increased by 14.7% ($p \leq 0.05$) and 14.7% ($p \leq 0.05$), respectively. In contrast, no significant changes were observed in the BAI-treated group ($p \leq 0.48$). We can conclude that only T3 could protect animals from CKD-induced anemia (**Table 4)**.

T3 but not BAI modulates mineral bone disorders in CKD

CKD-induced mineral bone disorders include abnormalities in bone and mineral composition [57]. CKD patients develop an inadequate 1,25-dihydroxyvitamin D level, predisposing them to

hyperphosphatemia. Furthermore, both processes together cause a reduced serum calcium level [5]. As a result of bone wear and tear, the serum alkaline phosphatase (ALP) level is increased in CKD patients [58–60]. Hence, serum phosphate, calcium, vitamin D3, and alkaline phosphatase (ALP) activities were estimated to investigate the potential of treatments for modulating mineral and bone disorders. Our results revealed a significant increase in the serum phosphate concentration and a decrease in the calcium concentration in the placebo-treated mice compared with those in the control mice (**Table 5)**.

Moreover, these changes in Ca and Pi levels during CKD were reversed toward their normal range by the T3 and BAI treatments, with maximum reversal caused by the combined treatment. Similarly, an increase in the mRNA expression of the phosphate channel NPT2a, followed by a decrease in the expression of the calcium channel TRPV5, was detected in CKD animals. However, the combined treatment had a much greater positive effect on the altered changes caused by CKD, reversing the abnormality through decreased NPT2a and increased TRPV5 mRNA expression **(Figure 9)**. Compared with those in control mice, a significant decrease in serum vitamin D3 levels and an increase in alkaline phosphatase (ALP) activity were also observed in placebo-treated mice, indicating bone wear and tear. However, these abnormal changes were counteracted by T3 and combined (T3 + BAI) treatment. No significant differences were observed in the BAI-treated group ($p \leq 0.34$) (**Table 5)**.

Protective effect on CKD-associated cardiovascular risk

End-stage renal disease (ESRD) is widely known to increase cardiovascular risk, and it is estimated that dialysis patients have cardiovascular death rates that are ten to one hundred times greater than those in the general population [61]. A heightened proinflammatory microenvironment and other modifying factors (dyslipidemia) during CKD predispose individuals to cardiovascular events **(Supplementary Result S3)**. The atherogenic index (AI) and coronary risk index (CRI), which are calculated on the basis of serum triglyceride, total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels (**Supplementary Table 1)**, were significantly greater ($p \leq 0.001$) in the placebo group than in the control group **(Figure 10a, b)**. The risk levels pertaining to the AI and CRI were lower in the T3- and BAI-treated animals than in the placebo**-treated animals. Compared with either of the individual treatments, the combined treatment was better at lowering the AI and CRI predispositions.**

Like kidney tissue, heart tissue was also checked for damage via H&E staining of thin sections. We found that in the placebo group, there was a significant increase in fibrosis and

inflammatory infiltration compared with those in the control group. Surprisingly, individual T3 and BAI treatments reduced fibrosis, and T3 had a greater antifibrotic effect than BAI did. Moreover, the combined treatment (T3 + BAI) produced a significantly more pronounced antifibrotic effect than either of the individual treatments did **(Figure 10c, d)**. Like those in the kidney, a similar trend of antioxidant enzyme activities in the heart tissue was observed, wherein SOD and catalase activities were significantly reduced in the placebo-treated mice ($p \leq 0.001$). In contrast, T3 and BAI alleviated the activity of both enzymes, and compared with the placebo, the combined treatment (T3 + BAI) resulted in the highest activity of both enzymes (**Table 25)**.

Discussion:

In the present study, we aimed to decipher the protective effect of combined T3 and BAI treatment on adenine-induced CKD in old mice. As per our previous findings [19,62], we intend to bidirectionally target CKD using the thyroid hormones T3 and BAI. T3 was used with the intention of maintaining a normal HPT axis in CKD, whereas BAI was used to counteract the exaggerated upregulation of the Wnt/β-catenin pathway, as previously reported. Interestingly, we found that both treatments could enhance Klotho via different targets. The details of the bidirectional treatment are illustrated in **Figure 11.** Furthermore, as CKD predisposes individuals to other complications, such as anemia, dyslipidemia, cardiovascular disease, and mineral bone disorders, we intend to evaluate the therapeutic potential of individual (either T3 or BAI) versus combined (T3 + BAI) treatment for these complications.

Feeding animals with a requisite concentration of adenine for a long duration is a chemically induced experimental model for chronic renal failure (CRF) [63]. Thus, adenine was used to induce CKD, as described by Rahman et al. (2018) [64]. The successful induction of CKD was confirmed by a significant increase in the serum creatinine level and a drastic reduction in the body weight of the treated animals. Following CKD induction, the mice were divided into different groups and given individual (T3 or BAI) treatments or combinations (T3 and BAI). We found that both T3 and BAI increased body weight, which provides the first clue as to how much the overall health parameters of CKD mice have improved. In contrast, the animals that received the combination therapy had a more significant increase in body weight. According to some reports, adenine-induced CKD in mice results in skeletal and cortical bone loss, especially in males [65]. This implies that our treatments could counteract this damage, thereby leading to visible and remarkable improvements in body weight.

Serum and urine biomarkers, including creatinine, urea, BUN, and albumin, are used as standard clinical indices to assess kidney disorders leading to CKD [52,63,66,67]. The adenine oral dose significantly impaired kidney function, as evidenced by increased serum creatinine, urea, BUN, and urine albumin levels, followed by decreased urinary excretion of creatine and urea. In this study, when CKD mice were treated with T3, BAI, or their combination (T3 + BAI), all the abnormal serum parameters reverted toward their normal range, indicating that there were improvements in kidney function after the treatments.

Previously, researchers reported that CKD has a substantial impact on the HPT axis, resulting in impaired thyroid hormone metabolism. Similarly, the prevalence of subclinical hypothyroidism increases with decreasing GFR [5,68,69]. Moreover, the prevalence of subclinical hypothyroidism increases with age (in individuals aged 60 years or above), which is correlated with a decrease in the GFR with age [70–72]. Most commonly observed in CKD patients is a decrease in the serum T3 and T4 levels and an increase in the TSH level [7]. Our findings corroborate earlier reports, where we observed that CKD mice had lower T3 levels and significantly higher serum TSH levels.

Hence, to correct the dysregulation of the HPT axis, exogenous T3 treatment was carried out at a dose reported previously [36,37]. Mice received exogenous T3 to prevent their HPT axis from becoming dysfunctional. After 12 h of T3 treatment, significantly lower TSH levels were observed only in the T3 and T3 + BAI groups. In contrast, no significant changes in the thyroid profile were observed in BAI-treated animals. The biological half-life of T3 and the projected rise in T3 levels led to this observation. The next stage of this study calls for more research into the ideal dosage and timing of the medication due to the design of the experiment's limitations in performing thyroid hormone pharmacokinetics in animals with established CKD (**Supplementary Results S1)**.

Glomerulosclerosis, interstitial fibrosis, and tubular atrophy are symptoms of renal fibrosis resulting from the inability of kidney tissue to be repaired after long-term, chronic damage [73]. Renal cells are replaced by ECM in the glomeruli and interstitium as CKD progresses, which is accompanied by the loss of renal cells [74,75]. Hence, we checked for morphological changes in the kidney through H&E and Masson's trichrome staining. An increase in inflammatory infiltration and protein casts was observed in CKD kidneys. Similarly, there was increased ECM deposition, as characterized by Masson's trichrome staining, after adenine treatment. Even if T3 and BAI therapy could reduce the degree of damage, the rate of improvement was modest. In contrast, a greater rate of matrix remodeling was observed in the combination treatment group, as evidenced by a considerable decrease in the fibrotic area and ECM

deposition. These findings indicate that combination (T3 + BAI) therapy in CKD mice has a more significant effect on preventing further deterioration of the kidney from CKD.

Klotho is expressed mainly on the cell surface of the DCT and PCT of the kidney [76–80]. Under normal physiological conditions, the kidney maintains normal Klotho levels [81–83]. However, a decrease in Klotho levels in individuals and animal models of CKD is evident in nephritic dysfunctions [84,85]. The mRNA expression of M-Kl and Sp-Kl decreases in CKD kidneys. Similar results were found for α-Kl and S-Kl protein expression, which was reduced upon CKD induction. All the impaired Klotho mRNA and protein expression caused by CKD reverted toward the normal range after T3 and BAI treatment but was not greater than that of the combination treatment. As reported previously, we speculate that the increased levels of Klotho observed in BAI may be exerted via its inhibition of the Wnt/β-catenin pathway, whereas T3 may directly activate Klotho, as evidenced in our previous report.

Furthermore, the ultimate standard route for various CKDs, including renal tubulointerstitial fibrosis, triggers end-stage renal disease (ESRD) development. Through its intersection with crucial signaling pathways, including the TGF-1 signaling pathway, the significance of GSK3β in the profibrogenic plasticity of renal tubular cells during CKD progression has recently been revealed [47]. According to other reports, proteinuric glomerulopathies and podocyte damage are just two examples of kidney diseases linked to elevated expression of GSK-3β in glomeruli and renal tubular cells [86–88]. Therefore, determining whether GSK3β expression changes after treatment is crucial. We found that GSK-3β protein expression in CKD kidneys considerably increased, indicating tubular inflammation and damage. Although T3 and BAI can reduce these aberrant GSK-3β levels, combination therapy had the most significant effect. These findings imply that protection from tubular damage can be attained by increasing the Klotho level bidirectionally.

The canonical Wnt/β-catenin pathway, an evolutionarily conserved signaling pathway, controls the pathogenesis of many diseases. Overexpression of this pathway has been observed in different types of kidney injury [89–92]. Sustained activation of the canonical Wnt/β-catenin pathway drives acute kidney injury (AKI) to CKD progression [93]. Similarly, we observed that adenine-fed animals more robustly activated the Wnt/β-catenin pathway, which was demonstrated by increased mRNA expression of the different Wnt ligands, decreased protein expression of CK-1 and increased production of the β-catenin protein in the kidney. The natural compound BAI effectively reduced the increased mRNA expression of all the Wnt ligands in CKD mice. Additionally, T3 and BAI effectively reversed the impaired expression of β-catenin and CK-1 in CKD mice, with combination therapy leading to greater β-catenin degradation.

We suggest that the effect of T3 on Wnt inhibition may be due to its impact on increased Klotho expression, as Klotho can inhibit the Wnt/β-catenin pathway without altering Wnt mRNA expression [94–96]. Similarly, since maximum levels of Klotho expression were observed in the combined treatment (T3 + BAI), we speculate that by inhibiting the Wnt/β-catenin pathway, BAI could prevent this major upregulated pathway, as observed in CKD, from inhibiting Klotho expression. Thus, steady upregulation of Klotho protein is maintained in the kidney, which results in pronounced beneficial effects.

Several studies have also shown that TGF-β is one of the most important profibrotic regulators of renal fibrosis in the development of CKD. It promotes matrix protein accumulation to produce ECM, prevents matrix breakdown, and regulates myofibroblast activation [97–100]. Numerous therapeutic approaches involving the inhibition of TGF-β have been studied in experimental models of CKD and in clinical trials on the function of TGF-β. These include the administration of antibodies that neutralize TGF-β and small interfering RNAs that target the TGF-β type II receptor, all of which can reduce structural renal injury and renal fibrosis in CKD [101,102]. Another finding in renal cultured epithelial cells is that Klotho inhibition enhances TGF-β1 expression, whereas TGF-β1 reduces Klotho expression, suggesting that decreased Klotho expression promotes TGF-β1 activity and that Klotho depletion is both a cause and a consequence of renal fibrosis in CKD [103]. In our current study, adenine-induced CKD mice presented increased TGF-β mRNA expression. This overexpression correlates with the upregulation of the Wnt/β-catenin pathway, culminating in decreased Klotho expression. However, a significant decrease in the level of TGF-β was observed in the combination treatment group. Our findings corroborate reports elsewhere [104], where Klotho can inhibit TGF-β signaling, which can be beneficial in suppressing renal fibrosis activity.

Additionally, Klotho can adversely regulate NF-κB, as it is an anti-inflammatory modulator that reduces proinflammatory gene transduction [105]. The PDLIM2/NF-B pathway is also inhibited by excess Klotho, which reduces the production of TNF-α, IL-6, and IL-12 and improves cyclosporine A-induced nephropathy in both in vivo and in vitro models [106]. Increased levels of inflammatory cytokines such as NF-κB, TNF-α, and interleukins have been reported in adenine-induced CKD mice. Similarly, in our study, NF-κB and IL-6 protein expression dramatically increased in the kidneys of CKD mice. According to our earlier findings, the anti-inflammatory effects of T3 and BAI followed a similar pattern, with a more notable decline observed in the group that received combination therapy (T3 + BAI). This result may be linked to the considerable increase in Klotho expression caused by the combination treatment.

Overproduction of oxidizing free radicals and inadequate antioxidant breakdown of these pro-oxidants lead to oxidative stress. Owing to reduced antioxidant activity and exponential ROS formation, CKD is a condition characterized by significant oxidative stress [107]. There is a strong correlation between the generation of ROS and that of Klotho. To combat oxidative stress, Klotho reportedly downregulates insulin/IGF-1/PI3K signaling, activating FoxO and inducing the formation of MnSOD [108]. Furthermore, intracellularly, higher levels of free radicals such as H_2O_2 and $O2^{\bullet-}$ are generated during the conversion of hypoxanthine/xanthine to uric acid by xanthine oxidase (XO) [109–111]. The overproduction of hypoxanthine reportedly triggered the overexpression of XO by adenine. This leads to oxidative damage in adenine-induced CKD kidneys [112]. Hence, we determined the kidney's antioxidant enzymes, such as catalase, and SOD activity. As expected, adenine-induced CKD kidneys presented reduced expression of both antioxidant enzymes. All our treatments were able to reduce the altered antioxidant enzyme activities (**Supplementary Results S2)**.

Anemia in CKD patients can result from multiple mechanisms, including hypothyroidism and shortened red blood cell survival. However, the most critical cause of CKD-induced anemia is decreased erythropoietin synthesis [5,113]. In the present study, the reduced hematocrit percentage and hemoglobin concentration observed indicate the occurrence of anemia in CKD mice. The anemia was ameliorated by treatment with T3 only, whereas BAI did not have any effect. This protective effect could be due to the well-known stimulatory effect of T3 on erythropoiesis [114–116].

Through the decreased excretion of inorganic phosphate (Pi), which is caused by a low level of 1,25-dihydroxyvitamin D, CKD is also linked to mineral and bone diseases. This decreased the serum Ca^{2+} concentration [117,118]. On the other hand, Klotho overexpression can reduce calcification in vivo, improve renal function, and increase phosphaturia [17]. Serum ALP is a biomarker for high-turnover bone disease, which usually increases in CKD patients [119]. Similarly, adenine-induced CKD animals presented significantly increased serum ALP activity and Pi levels, with decreased serum Vit-D3 and Ca^{2+} levels.

A reduction in ALP activity with increasing Vit-D3 levels was observed to be produced by T3 treatment only. In contrast, a protective effect in the form of Pi excretion and Ca^{2+} reabsorption was observed in both the T3- and BAI-treated groups. The regulation of the phosphate channel (NPT2a) and calcium channel (TRPV5) by Klotho has been described elsewhere [120,121]. Both T3 and BAI downregulated the mRNA expression of NPT2a and increased the mRNA expression of TRPV5, with a potential effect observed with their combined treatment. Hence, the phosphate and calcium channel mRNA data corroborate the serum Pi and Ca^{2+} profiles. The

observed effects on NPT2a and TRPV5 may involve increased Klotho expression caused by T3 and BAI.

Chronic kidney disease is a systemic pathology with dyslipidemia commonly observed among CKD patients. As renal function decreases, the incidence of hyperlipidemia increases. A high degree of elevated LDL cholesterol and triglyceride levels is proportional to the severity of renal impairment [5]. The beneficial effects of the treatment have also been observed systematically. For example, with respect to lipid profile parameters, the serum triglyceride, cholesterol, LDL-C, and VLDL-C levels increased significantly, with a decrease in HDL-C levels in CKD mice. The combined T3 and BAI treatment protected against CKD-induced dyslipidemia (**Supplementary Results S3)**.

Furthermore, dyslipidemia and elevated serum TSH levels are independent risk factors for cardiovascular diseases [122,123]. Therefore, we calculated the AI and CRI from the serum lipid profile. The risk factor for CVD was greater in CKD mice and was reduced after T3 and BAI treatment, with the lowest risk observed in animals that received the combined treatment. Additionally, as CKD mice presented a greater risk for CVD, we checked heart tissue for fibrosis via H&E staining. Surprisingly, CKD hearts also exhibited significant fibrosis. As we observed in the kidney, in heart tissue, the combined treatment provided greater protection than the individual treatments did, as indicated by a reduction in the fibrotic area, which is a clear indication of its protective effect against CVD. The increased oxidative stress in the kidney prompted us to assess antioxidant enzyme activity in heart tissue. Both catalase activity and SOD activity were reduced in CKD patients and increased after treatment, indicating the protective effect of Klotho, even in heart tissue.

Disclosure

All the authors declare that they have no competing interests and have consented to the submission of this manuscript.

Ethics approval: Approved by the Institutional Animal Ethics Committee (IAEC) of Pondicherry University, India (IAEC approval ref. No: PU/CAHF/23rd IAEC/2019/03, dated 15.04.2019

Acknowledgments

The authors acknowledge the Department of Biochemistry and Molecular Biology, Pondicherry University, India, and the Department of Science & Technology “Fund for Improvement of S&T Infrastructure”, (DST-FIST), Science and Engineering Research Board

(SERB), Government of India, for financial and overall support.

Credit authorship contribution statement

Saswat Kumar Mohanty: Conceptualization, methodology, investigation, formal analysis, writing - original draft, project administration

Vikas kumar Sahu, Bhanu Pratap Singh: Helped SKM in conducting investigations as a part of their M.Sc. dissertation.

Kitlangki Suchiang: Supervision, conceptualization, validation, formal analysis, review and editing, funding acquisition

Funding

This work was supported by the Department of Biochemistry and Molecular Biology, Pondicherry University, India, and SERB, New Delhi (File No EEQ/2022/000514). SKM was supported by a fellowship from the Council of Scientific and Industrial Research (CSIR) (File no 09/559(0128)/2019-EMR-I), Ministry of Science and Technology, Government of India.

Data availability

The datasets generated and/or analyzed in this study are available from the corresponding author Kitlangki Suchiang upon reasonable request.

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Tables:

Table 1.

	Primer Sequence 5' to 3'	
Gene	**Forward**	**Reverse**
MKL	CAATGGCTTTCCTCCTTTAC	AACACAGGTTTGCGTAGTCT
Sp-KL	CAATGGCTTTCCTCCTTTAC	GACTTCTGTGTTGAATCTTA
NPT2a	AGGTGAGCTCCGCCATTCCGA	CCCTGCAAAAGCCCGCCTGA
TRPV5	CTCCGCCTTCCATCGAAGTTC	GATGTCAGCTCCATGCTCAATG
WNT-1	GCCCTAGCTGCCAACAGTAGT	GAAGATGAACGCTGTTTCTCG
WNT-3	GGGGCGTATTCAAGTAGCTG	GTAGGGACCTCCCATTGGAT
WNT-8A	CTGACTACTGCAACCGCAAC	TGACAGTGCAACACCACTGA
WNT-8B	CCAGAGTTCCGGGAGGTAG	GAGATGGAGCGGAAGGTGT
WNT-10A	GCGCTCCTGTTCTTCCTACT	ATGCCCTGGATAGCAGAGG
TGFβ	CGAAGCGGACTACTATGCTAAA	TCCCGAATGTCTGACGTATTG
Beta Actin	CACACTGTGCCCATCTACG	GCCATCTCTTGCTCGAAGTC

Table 2.

	Control	Placebo	T3	BAI	T3 + BAI
Serum Creatinine (mg/dL)	0.263 ± 0.063	2.281 ± 0.093 ###	1.281 ± 081 *	1.541 ± 0.040 **	0.609 ± 0.034 **
Urine Creatinine (mg/dL)	17.34 ± 0.093	5.606 ± 0.393 ##	11.91 ± 0.343 ***	9.813 ± 0.375 **	13.88 ± 0.187 ***
Serum Urea (mg/dL)	54.66 ± 2.241	85.34 ± 2.586 #	62.93 ± 2.434 **	64.66 ± 1.96 **	52.59 ± 2.473 **
Urine Urea (mg/dL)	311.2 ± 4.31	150 ± 6.897 ##	233.6 ± 6.034 **	269.8 ± 7.759 ***	306 ± 5.674 ***
BUN (mg/dL)	25.58 ± 1.049	39.94 ± 1.211 #	29.45 ± 1.324 **	30.26 ± 0.403 **	24.61 ± 1.243 **
Urine Albumin (g/dL)	35.85 ± 1.887	109.4 ± 3.774 ###	52.83 ± 3.656 **	62.26 ± 1.887 **	39.62 ± 1.764 **

The values are mean ± SEM. n = 5. *, **, and *** indicate the significance level $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively from placebo. #, ##, and ### indicate the significance level $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively from control.

Table 3.

	Criteria				Results				
Type	**0**	**1**	**2**	**3**	**Control**	**Placebo**	**T3**	**BAI**	**T3 + BAI**
Inflammatory Infiltration	None	Scatter	Focal	Diffuse	0	3	2	2	1
Protein Cast	None	< 10%	10-25%	> 25%	0	2	1	1	1
Interstitial Fibrosis	None	< 25%	25-50%	> 50%	0	3	2	2	1
ECM Deposition	None	< 25%	25-50%	> 50%	0	3	1	2	1
				Total	**0**	**11**	**6**	**7**	**4**

Each sample was performed based on ten randomly selected fields per section under x400 magnification.

Table 4.

	Control	Placebo	T3	BAI	T3 + BAI
Hematocrit %	45.93 ± 0.961	35.30 ± 0.6580 #	41.32 ± 0.607 *	34.93 ± 0.809 ns	40.86 ± 0.455 *
Hemoglobin (mg/dL)	15.0 ± 0.300	11.5 ± 0.250 #	13.2 ± 0.200 *	11.0 ± 0.300 ns	13.1 ± 0.200 *

The values are mean ± SEM. n = 5. * indicate the significance level $p \leq 0.05$ from placebo. # Indicate the significance level $p \leq 0.05$ from control. ns represent not significant

Table 5.

	Control	Placebo	T3	BAI	T3 + BAI
1,25, dihydroxy vitamin D3 (ng/dL)	6.679 ± 0.27	3.691 ± 0.206 #	5.683 ± 0.0364 **	4.043 ± 0.0435 ns	5.786 ± 0.211 **
ALP activity (U/L)	12.8 ± 0.917	39.9 ± 1.38 ##	17.4 ± 1.83 **	35.8 ± 1.38 ns	18.3 ± 2.75 **
Inorganic phosphate (mg/dL)	6.06 ± 0.275	10.8 ± 0.333 ##	7.95 ± 0.201 **	8.22 ± 0.295 **	6.20 ± 0.239 **
Calcium (mg/dL)	3.31 ± 0.180	1.14 ± 0.0601 ##	2.25 ± 0.132 *	1.95 ± 0.0721 *	2.75 ± 0.252 **

The values are mean ± SEM. n = 5. *, and ** indicate the significance level $p \leq 0.05$, and $p \leq 0.01$, respectively from placebo. #, and ## indicate the significance level $p \leq 0.05$, and $p \leq 0.01$, respectively from control.

Figures:

Fig. 1.

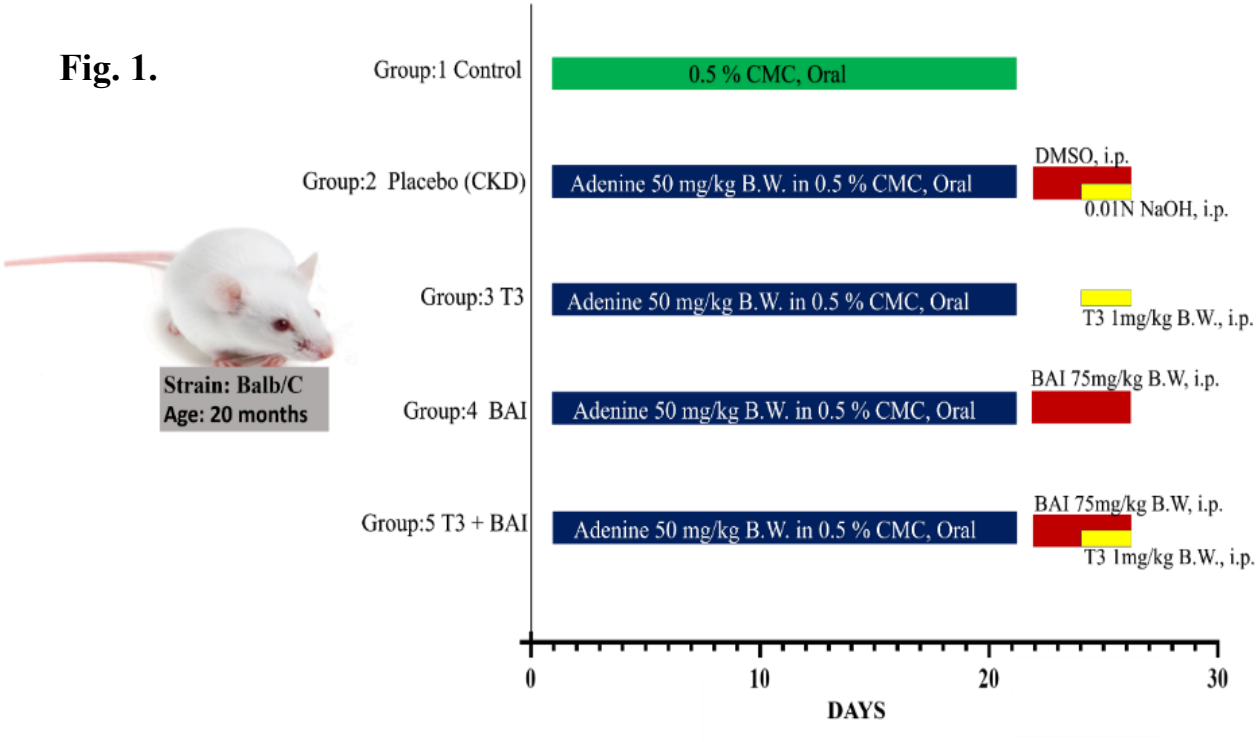


Fig. 2.

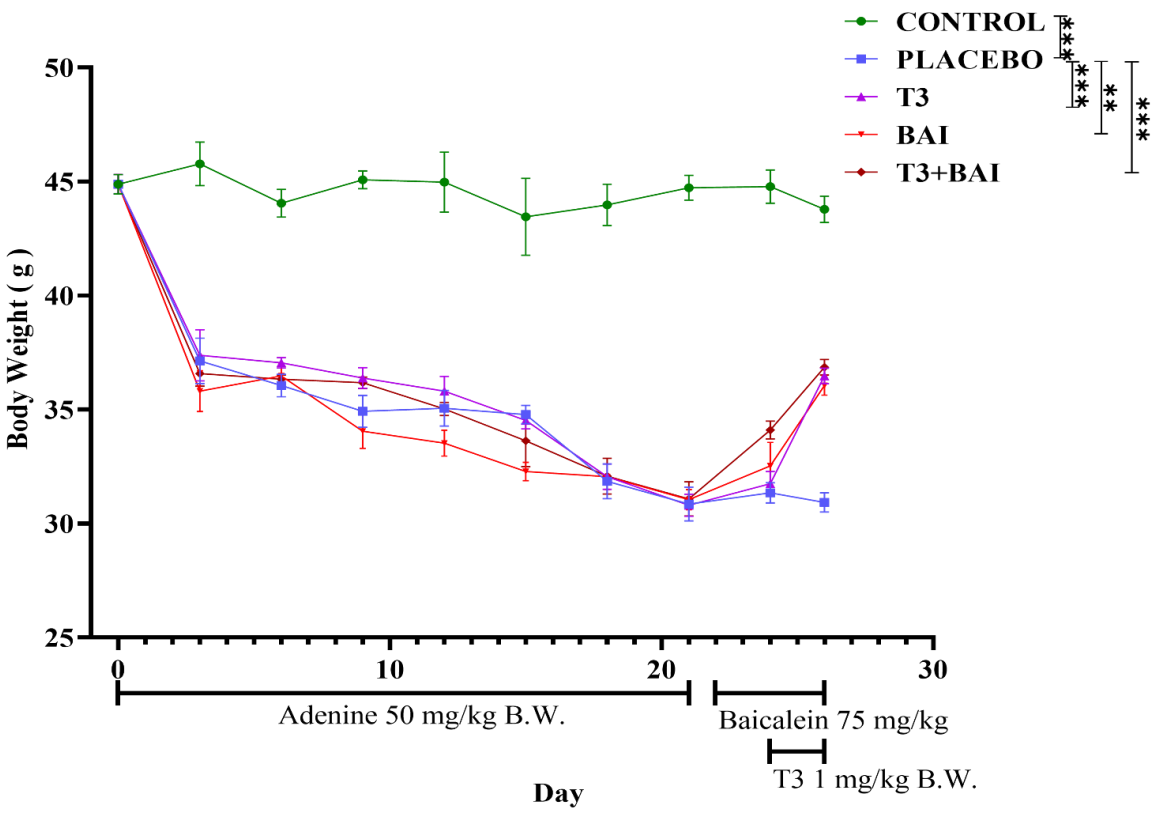


Fig. 3.

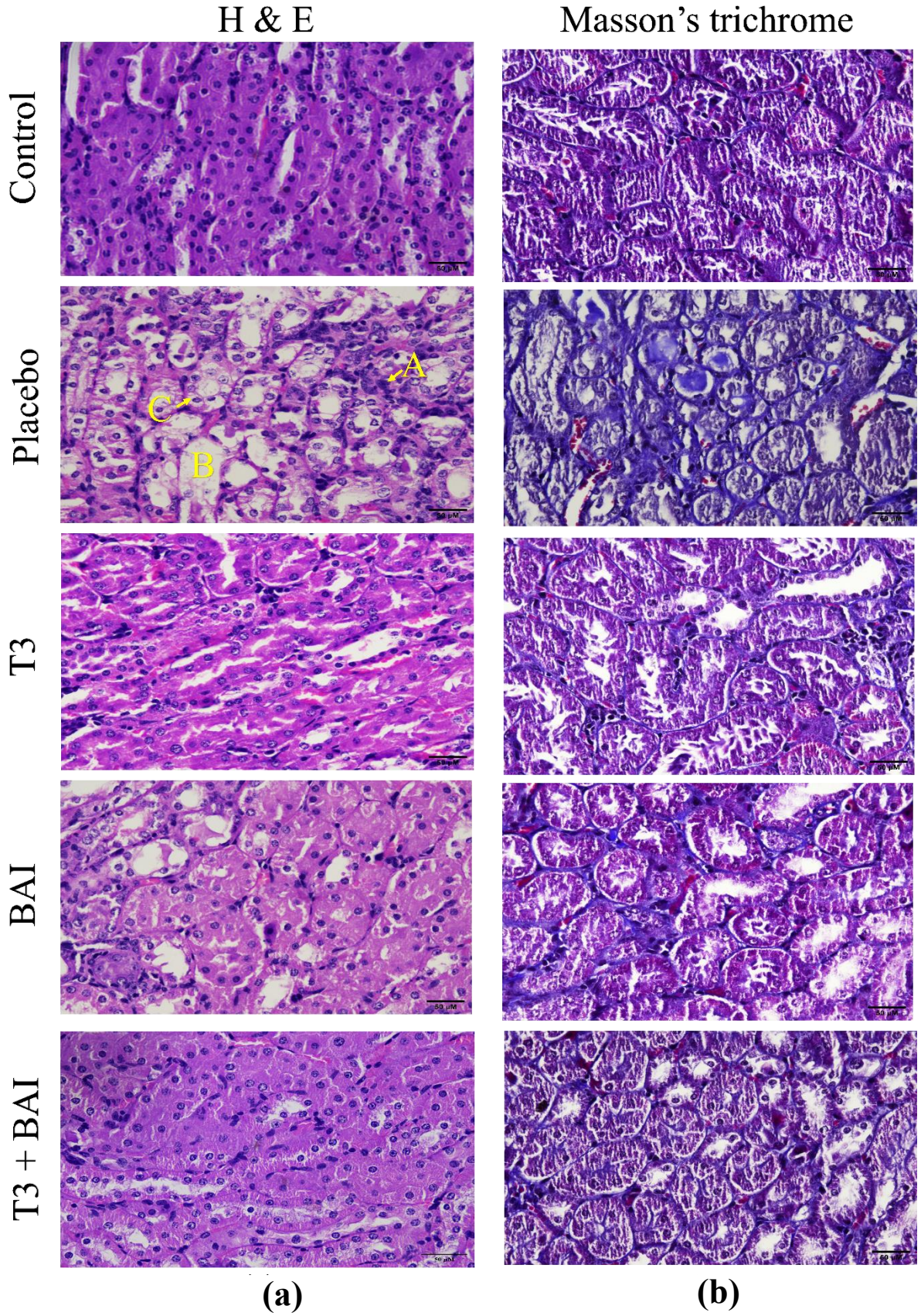
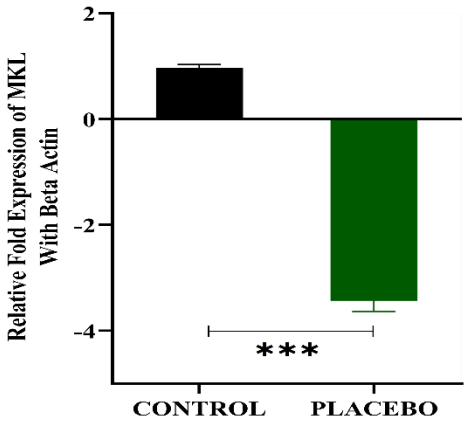
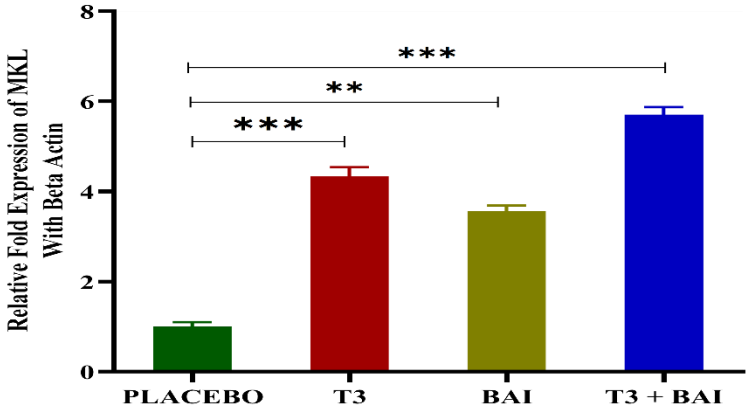


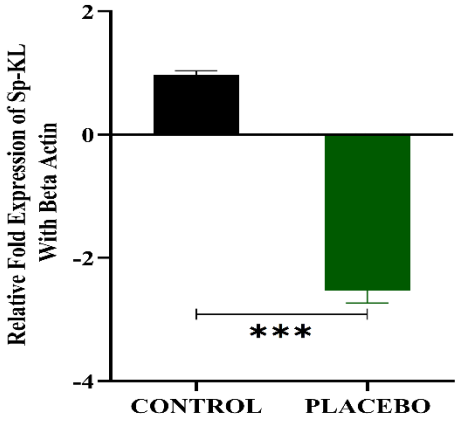
Fig. 4.


Relative Fold Expression of MKL With Beta Actin
2
0
-2
-4
***
CONTROL
PLACEBO

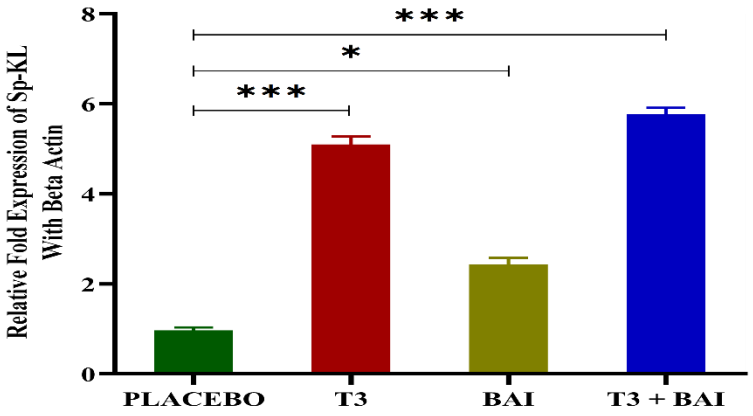
(a)


Relative Fold Expression of MKL With Beta Actin
8
6
4
2
0
***
**
***
PLACEBO
T3
BAI
T3 + BAI

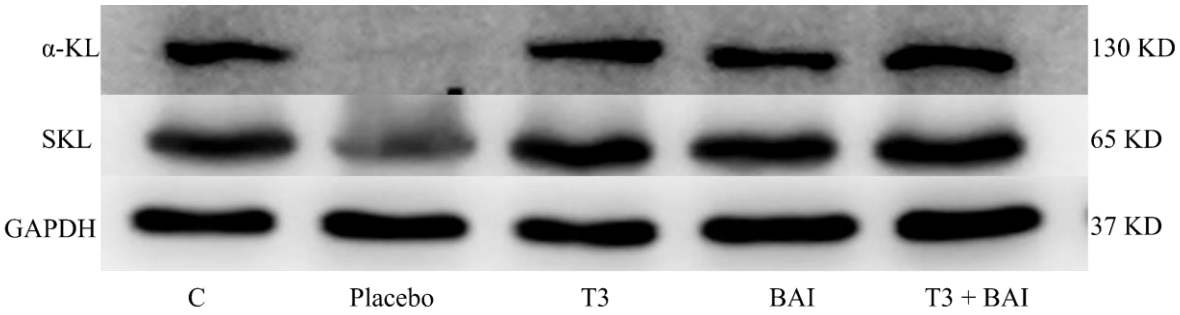
(b)


Relative Fold Expression of Sp-KL With Beta Actin
2
0
-2
-4
***
CONTROL
PLACEBO

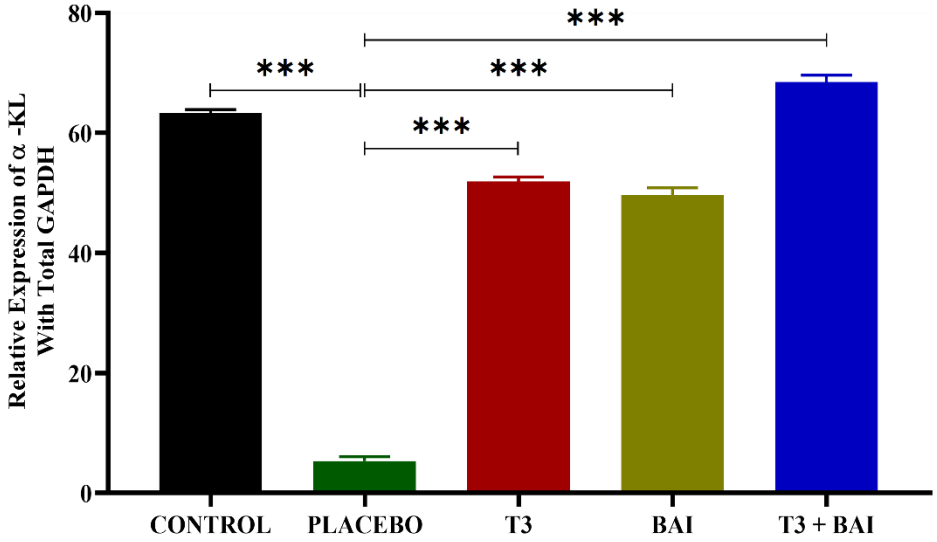
(c)


Relative Fold Expression of Sp-KL With Beta Actin
8
6
4
2
0
***
*
***
PLACEBO
T3
BAI
T3 + BAI

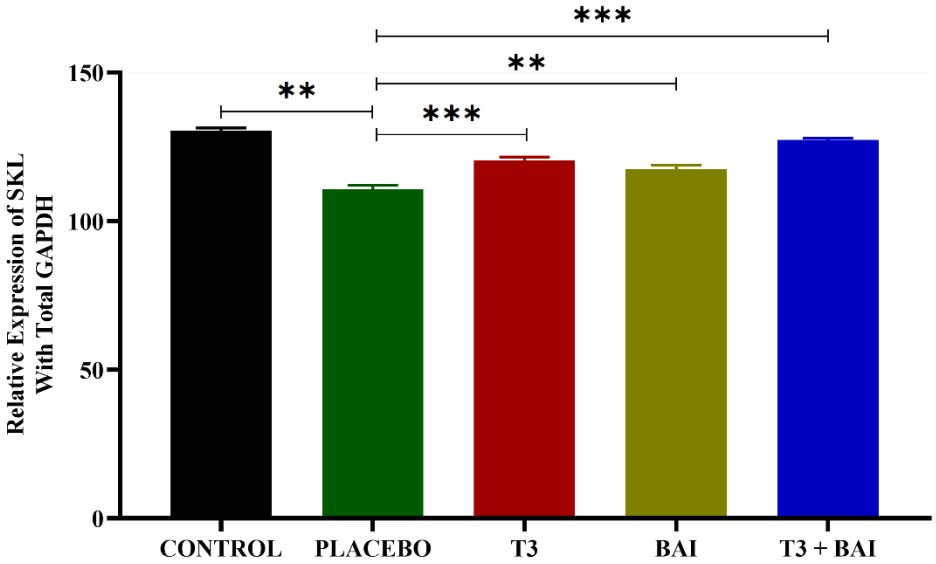
(d)


α-KL
SKL
GAPDH
130 KD
65 KD
37 KD
C
Placebo
T3
BAI
T3 + BAI

(e)

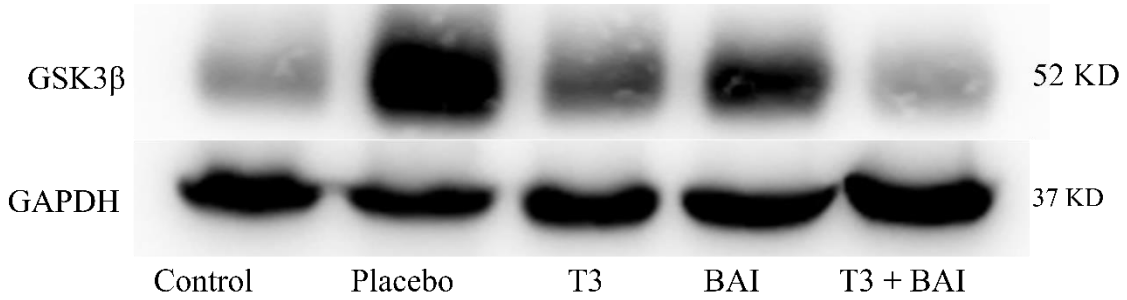

Relative Expression of α -KL
With Total GAPDH
80
60
40
20
0
***
***
***
***
CONTROL
PLACEBO
T3
BAI
T3 + BAI

(f)

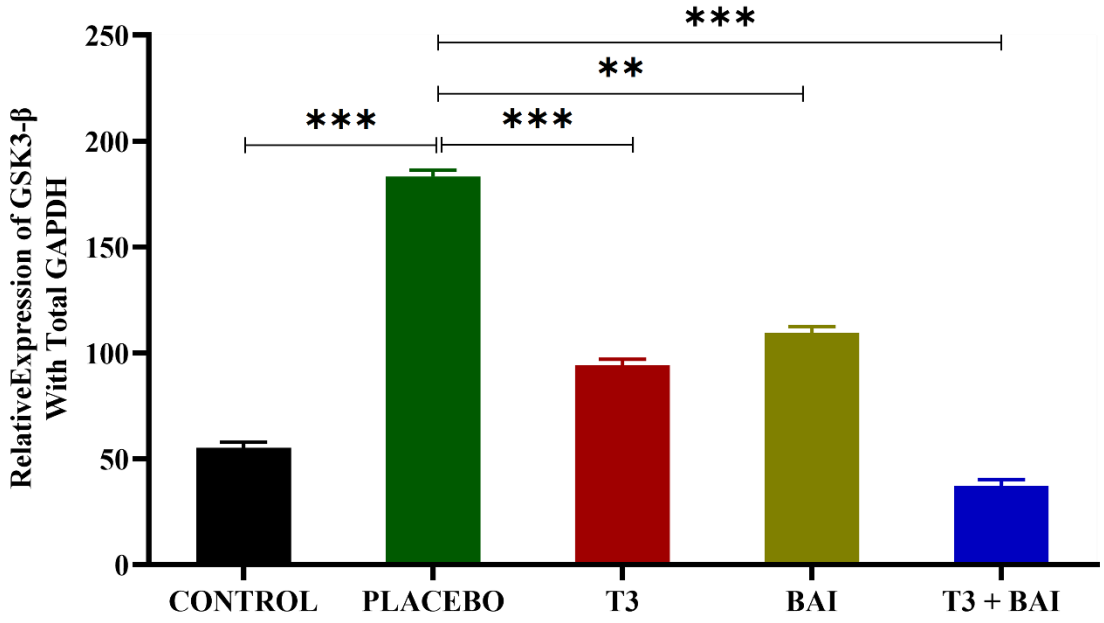

Relative Expression of SKL
With Total GAPDH
150
100
50
0
**
***
**
***
CONTROL
PLACEBO
T3
BAI
T3 + BAI

(g)

Fig. 5.

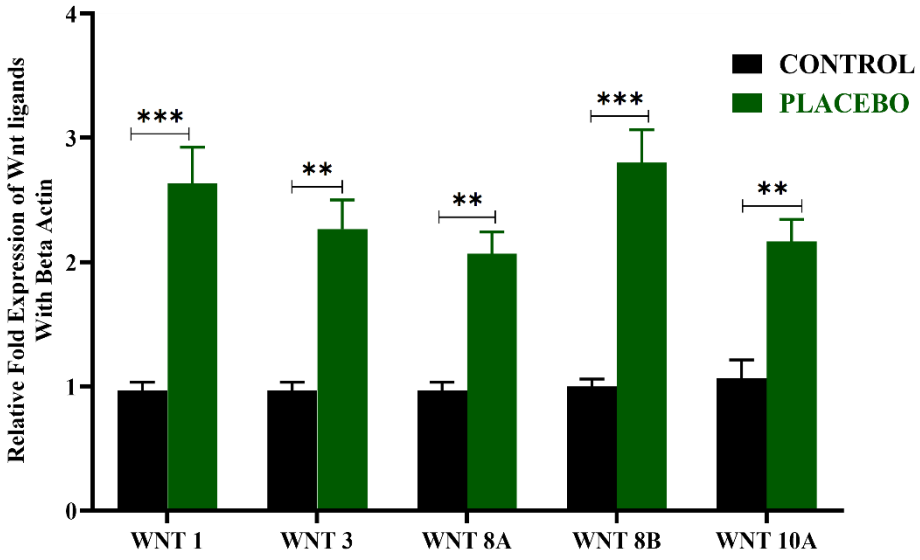

GSK3β
52 KD
GAPDH
37 KD
Control
Placebo
T3
BAI
T3 + BAI

(a)

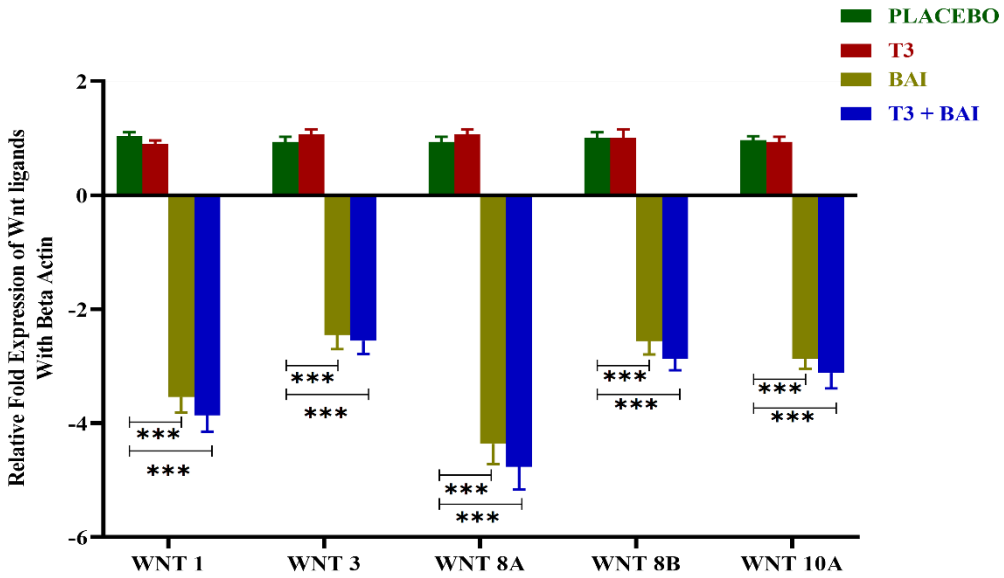

RelativeExpression of GSK3-β
With Total GAPDH
250
200
150
100
50
0
***
**
***
***
CONTROL
PLACEBO
T3
BAI
T3 + BAI

(b)

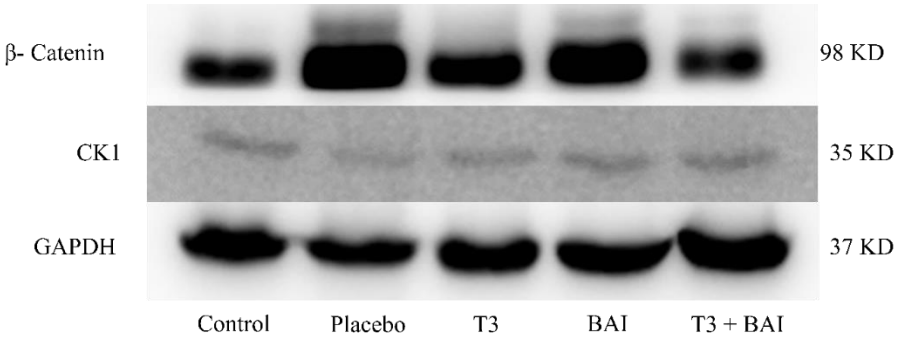
Fig. 6.


Relative Fold Expression of Wnt ligands With Beta Actin
CONTROL
PLACEBO
***
**
**
***
**
WNT 1
WNT 3
WNT 8A
WNT 8B
WNT 10A

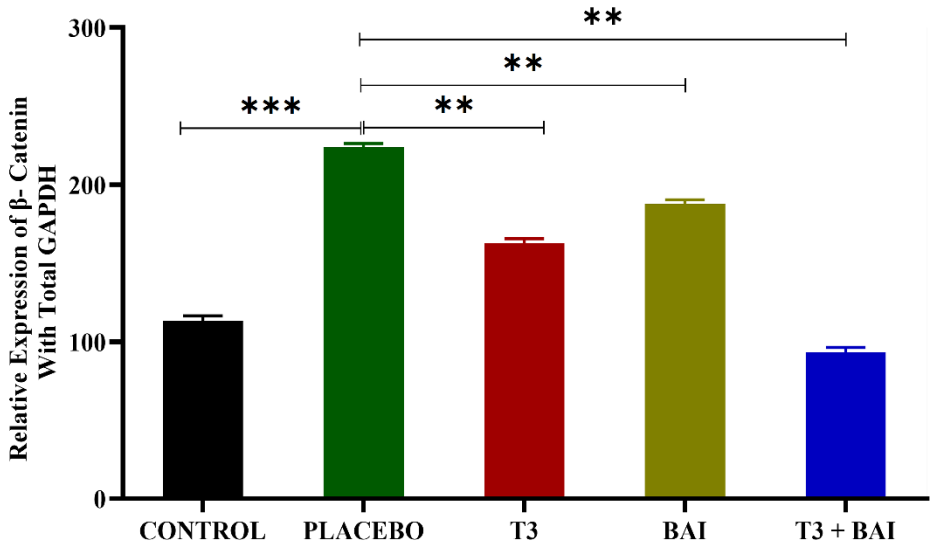
(a)


PLACEBO
T3
BAI
T3 + BAI
Relative Fold Expression of Wnt ligands With Beta Actin
***
WNT 1
WNT 3
WNT 8A
WNT 8B
WNT 10A

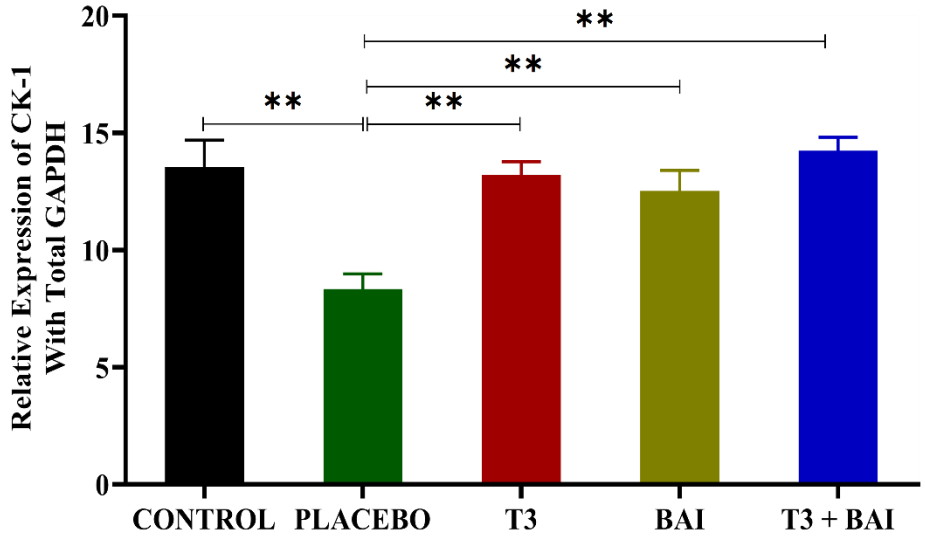
(b)


β- Catenin
CK1
GAPDH
98 KD
35 KD
37 KD
Control
Placebo
T3
BAI
T3 + BAI

(c)


Relative Expression of β- Catenin With Total GAPDH
300
200
100
0
***
**
**
**
CONTROL
PLACEBO
T3
BAI
T3 + BAI

(d)


Relative Expression of CK-1 With Total GAPDH
20
15
10
5
0
**
**
**
**
CONTROL
PLACEBO
T3
BAI
T3 + BAI

(e)

Fig. 7.

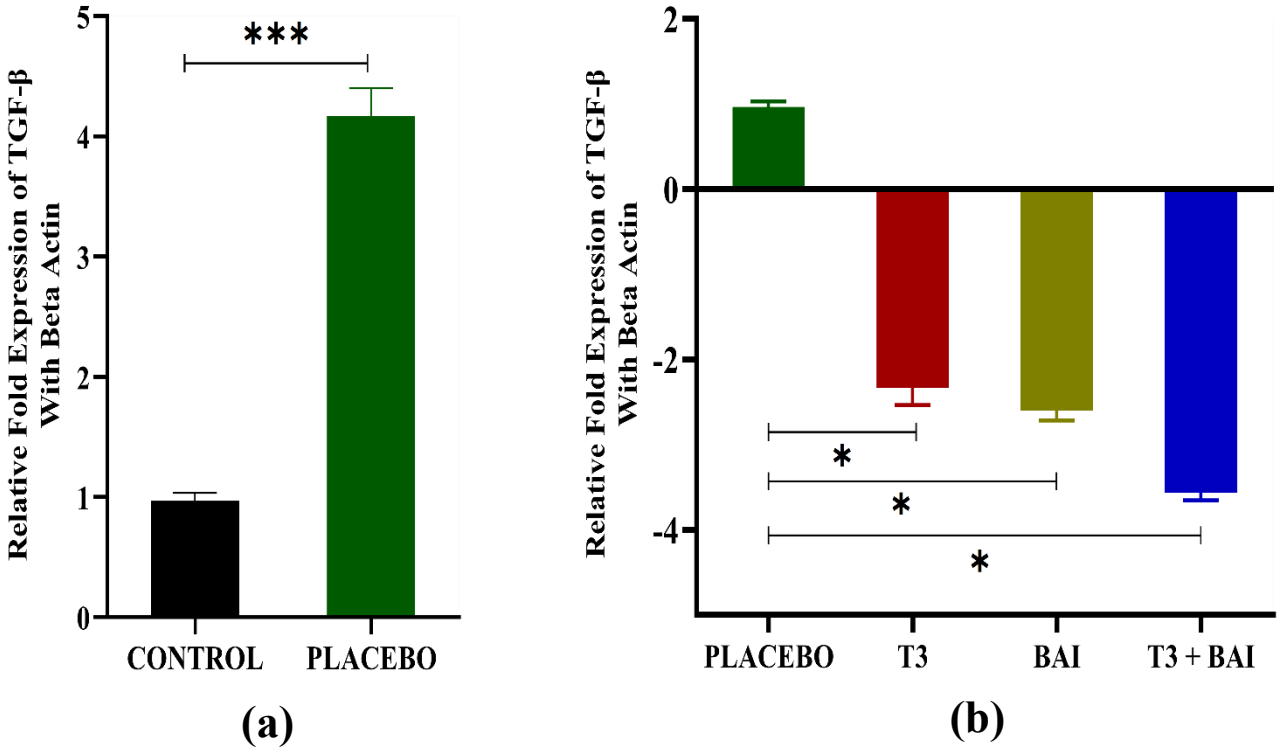

Relative Fold Expression of TGF-β With Beta Actin
5
4
3
2
1
0
***
CONTROL
PLACEBO
(a)
Relative Fold Expression of TGF-β With Beta Actin
2
0
-2
-4
*
*
*
PLACEBO
T3
BAI
T3 + BAI
(b)

Fig. 8.

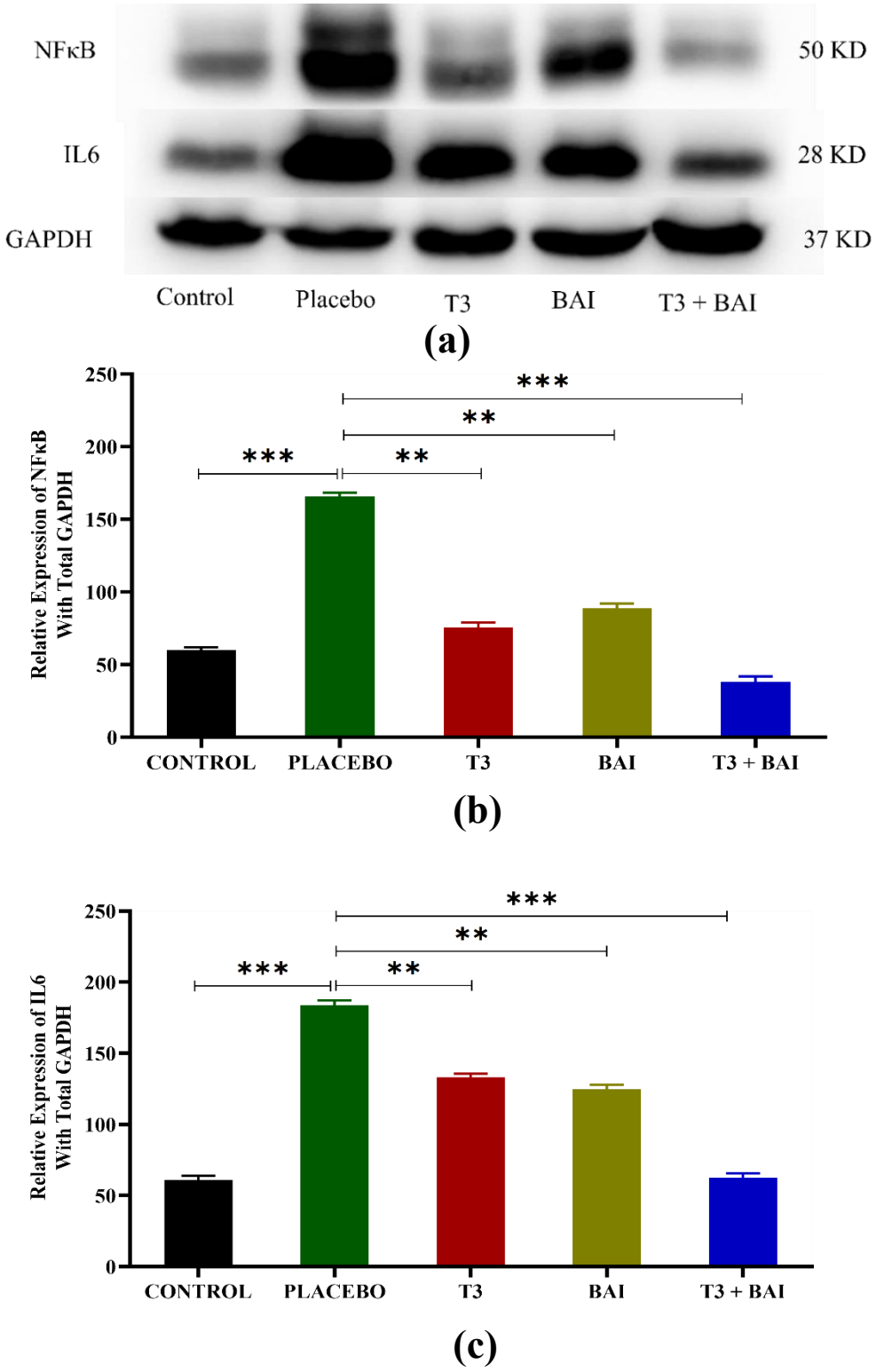

NFκB
IL6
GAPDH
50 KD
28 KD
37 KD
Control
Placebo
T3
BAI
T3 + BAI
(a)
Relative Expression of NFκB With Total GAPDH
250
200
150
100
50
0
***
**
***
**
CONTROL
PLACEBO
T3
BAI
T3 + BAI
(b)
Relative Expression of IL6 With Total GAPDH
250
200
150
100
50
0
***
**
***
**
CONTROL
PLACEBO
T3
BAI
T3 + BAI
(c)

Fig. 9.

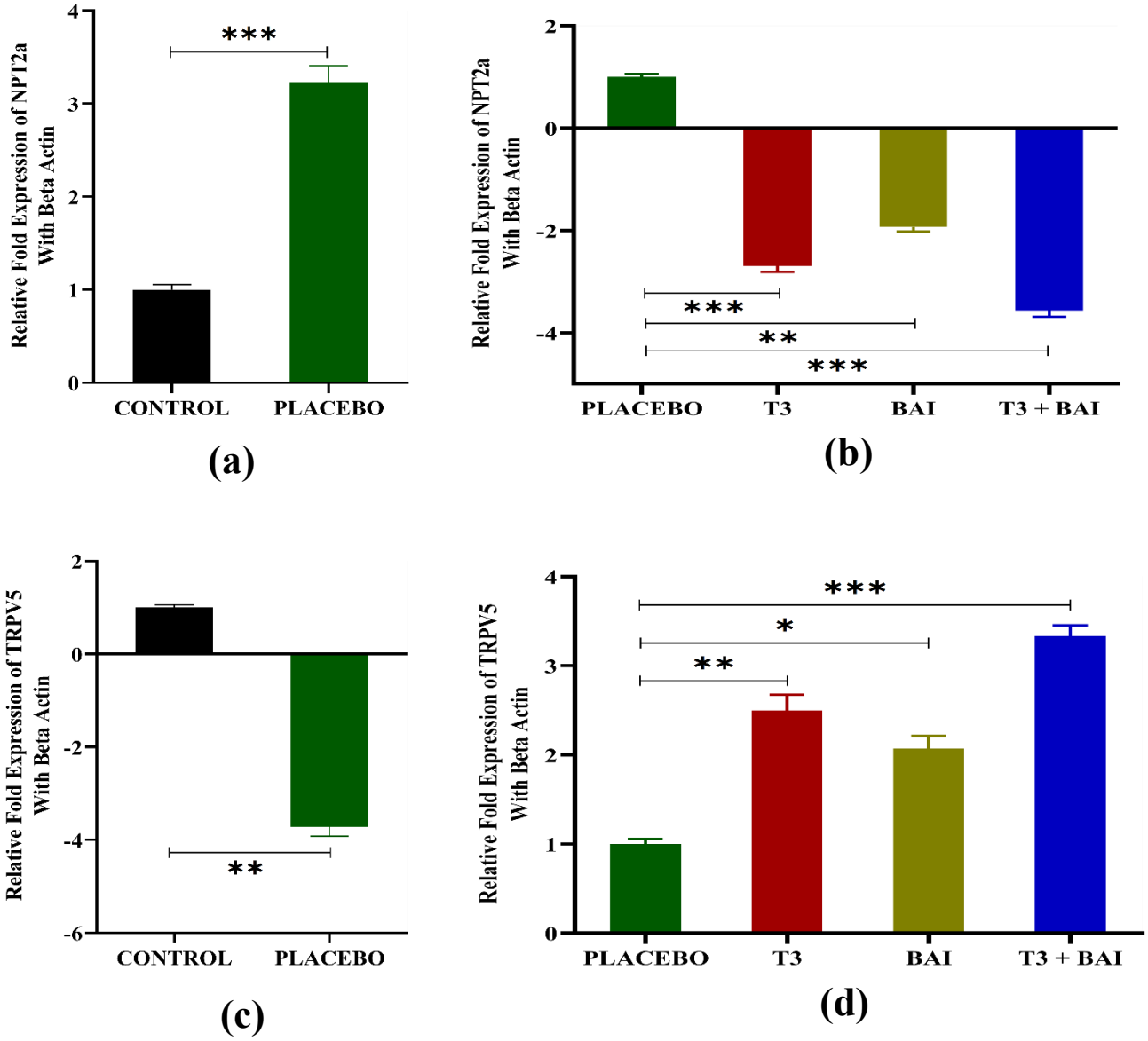

Relative Fold Expression of NPT2a With Beta Actin
***
CONTROL
PLACEBO
(a)
Relative Fold Expression of NPT2a With Beta Actin
***
**
***
PLACEBO
T3
BAI
T3 + BAI
(b)
Relative Fold Expression of TRPV5 With Beta Actin
**
CONTROL
PLACEBO
(c)
Relative Fold Expression of TRPV5 With Beta Actin
***
*
**
PLACEBO
T3
BAI
T3 + BAI
(d)

Fig. 10.

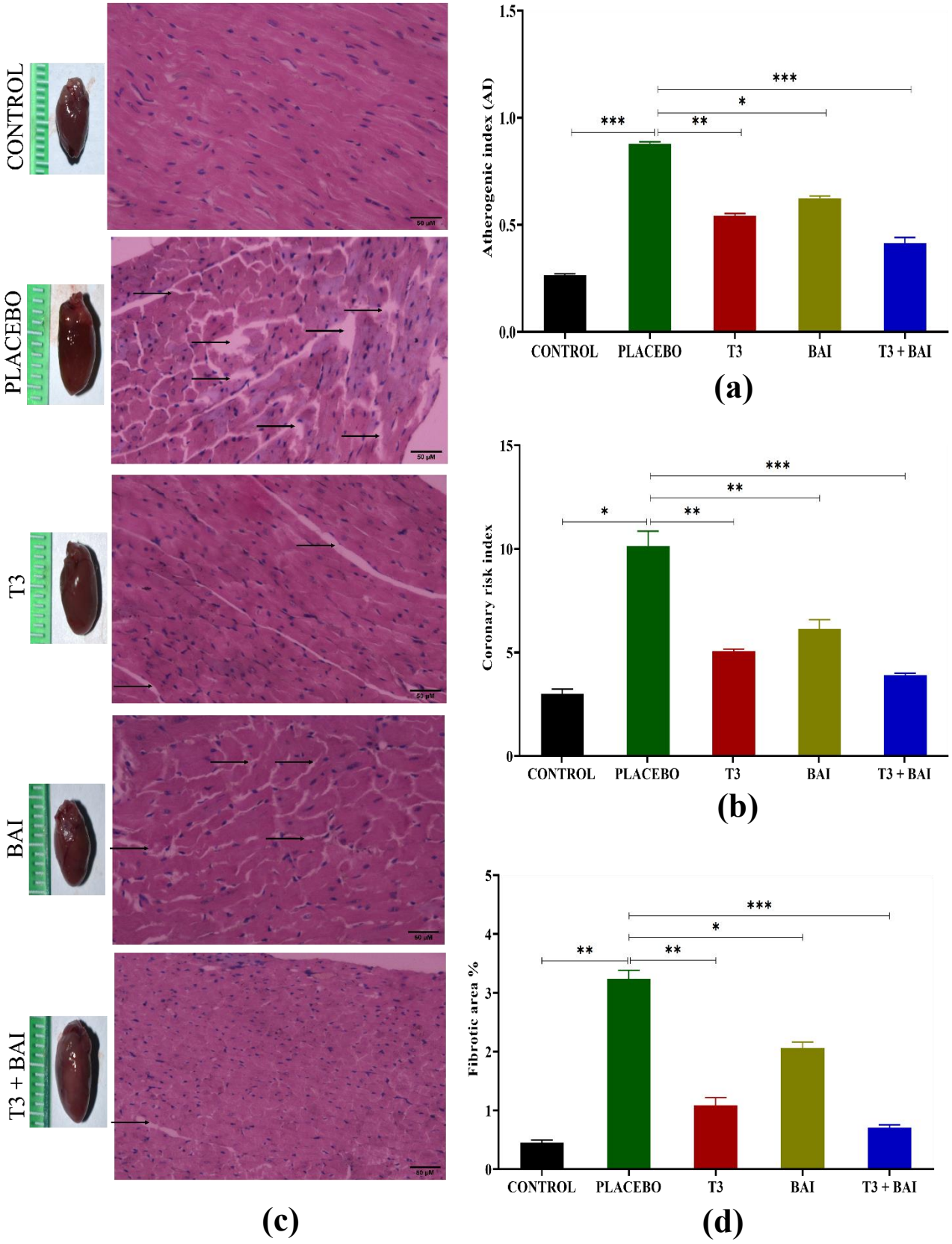

CONTROL
PLACEBO
T3
BAI
T3 + BAI
Atherogenic index (AI)
1.5
1.0
0.5
0.0
***
**
*
CONTROL
PLACEBO
T3
BAI
T3 + BAI
(a)
Coronary risk index
15
10
5
0
(b)
Fibrotic area %
5
4
3
2
1
0
(c)
(d)

Fig. 11.

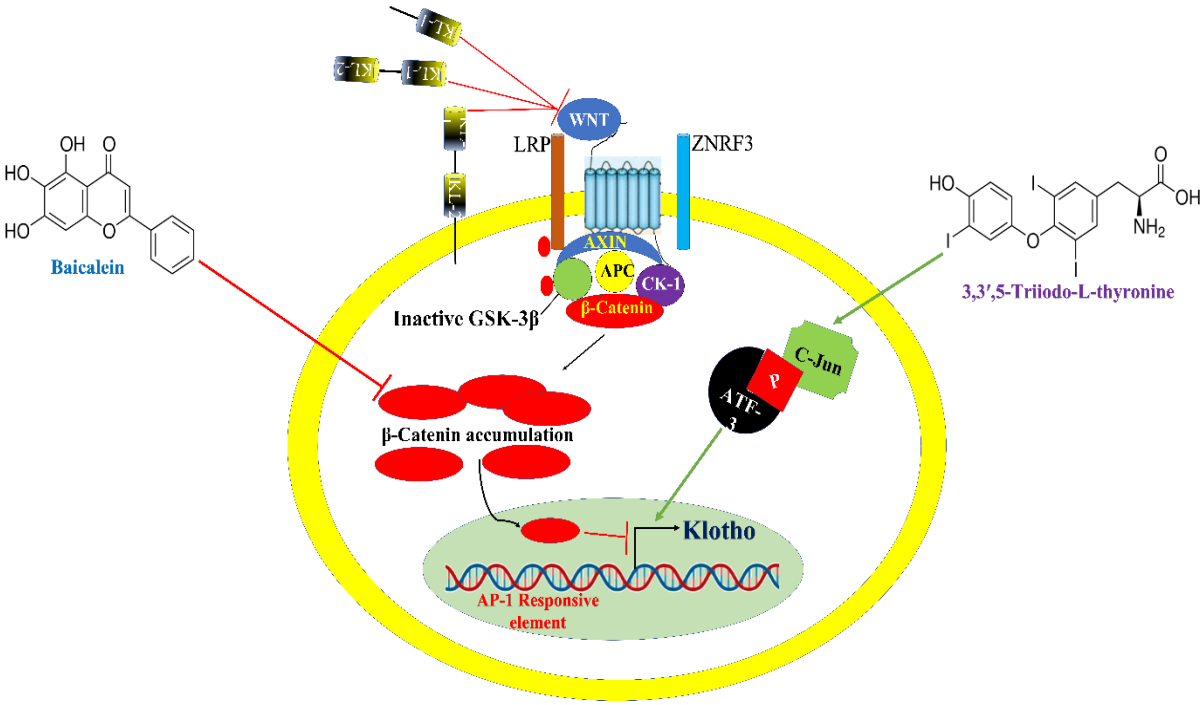


Figure Legends:

Fig. 1. Animal grouping and treatments.

Fig. 2. Body weight profile of BALB/c mice during CKD induction and treatment. Body weight was measured every 2 days during the treatment. A significant reduction in body weight was observed after adenine treatment. However, treatment with either T3, baicalein (BAI) or the combination (T3 + BAI) significantly increased the body weights of the animals. n = 10 per group. ∗∗ and ∗∗∗ indicate significance at $p \leq 0.01$ and $p \leq 0.001$, respectively.

Fig. 3. Analysis of renal pathological sections from CKD patients. These findings indicate the protective effects of T3, BAI, and their combination (T3 + BAI) against CKD-induced renal fibrosis. **(a)** Hematoxylin and eosin (H&E) staining of renal pathological sections (x400), **(b)** Masson's trichrome staining for extracellular matrix deposition analysis (x400). **A, B** and **C** represent inflammatory infiltration, protein casting, and cytoplasmic vacuolation, respectively. The scale bar represents 50 µM.

Fig. 4. Effects of T3, BAI, and the combined treatment (T3 + BAI) on Klotho expression in CKD kidneys. Klotho mRNA, i.e., membrane Klotho (M-KL) and spliced Klotho (Sp-KL), and protein, i.e., α-KL and S-KL, expression are decreased in CKD. After treatment with T3 and BAI in CKD mice, both the mRNA and protein expression levels increased significantly, with the combined treatment (T3 + BAI) resulting in the greatest increase in expression. **(a)** and **(b)** represent M-KL mRNA expression; **(c)** and **(d)** represent Sp-KL mRNA expression. **(e)** Western blot analysis of α-KL and soluble Klotho (S-KL); **(f)** and **(g)** Densitometric analysis of α-KL and S-KL, respectively. The data are expressed as the means ± SEMs from 3

independent experiments. ∗, ∗∗, and ∗∗∗ indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Fig. 5. Effects of T3, BAI, and the combination (T3 + BAI) on GSK-3β protein expression. T3 and BAI successfully reduced GSK-3β protein expression, with a greater reduction observed in the combined (T3 + BAI) treatment group. **(a)** and **(b)** Western blot analysis of GSK-3β and densitometric analysis in CKD mice. The data are expressed as the means ± SEMs from 3 independent experiments. ∗∗ and ∗∗∗ indicate significance at $p \leq 0.01$ and $p \leq 0.001$, respectively.

Fig. 6. Effects of T3, BAI, and the combined treatment (T3 + BAI) on the Wnt-β-catenin pathway in CKD kidneys. CKD mice presented increased Wingless-related integration site (Wnt) ligand mRNA expression with increased β-catenin protein expression. The downregulation of the expression of all the mRNAs and β-catenin proteins was observed in both the T3 and BAI treatment groups, with the maximum reducing effect observed in the combined treatment group. **(a)** and **(b)** mRNA expression of Wnt ligands in different groups. **(c)** Western blot analysis of β-catenin and creatine kinase 1 (CK-1) expression; **(d)** and **(e)** Densitometric analysis of β-catenin and CK-1 protein expression, respectively. The data are expressed as the means ± SEMs from 3 independent experiments. ∗∗ and ∗∗∗ indicate significance at $p \leq 0.01$ and $p \leq 0.001$, respectively.

Fig. 7. Effects of T3, BAI, or their combination (T3 + BAI) on TGF-β mRNA expression. The abnormal increase in transforming growth factor-β (TGF-β) mRNA expression was reduced by T3 and BAI treatment, with a greater reduction in the combined treatment. The data are expressed as the means ± SEMs from 3 independent experiments. ∗ and ∗∗∗ indicate significance at $p \leq 0.05$ and $p \leq 0.001$, respectively.

Fig. 8. Effects of T3, BAI, and the combined treatment (T3 + BAI) on proinflammatory marker levels in CKD kidneys. The protein expression of proinflammatory markers such as nuclear factor kappa B (NFκB) and interleukin 6 (IL-6) is significantly increased in CKD kidneys. In terms of kidney protection, T3 and BAI reduced both NFκB and IL-6 expression, with the greatest reduction observed in the combined treatment group. **(a)** Western blot analysis of NFκB and IL-6; **(b)** and **(c)** Densitometric analysis of NFκB and IL-6 protein expression, respectively. The data are expressed as the means ± SEMs from 3 independent experiments. ∗∗ and ∗∗∗ indicate significance at $p \leq 0.01$ and $p \leq 0.001$, respectively.

Fig. 9. Effects of T3, BAI, and the combined treatment (T3 + BAI) on the mRNA expression of phosphate and calcium transporters. T3 and BAI treatment significantly protected the kidney and altered mRNA expression, with a more pronounced effect in the combined treatment group. **(a)** and **(b)** mRNA expression of NPT2a in different groups and treatments. **(c)** and **(d)** represent the mRNA expression of TRPV5. The data are expressed as the means ± SEMs from 3 independent experiments. ∗, ∗∗, and ∗∗∗ indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Figure 10. Protective effect against CKD-induced cardiovascular disorder (CVD). T3 and BAI had protective effects against CKD-induced cardiovascular damage, with more pronounced effects observed with T3 treatment. However, maximum protection was observed in the combined (T3 + BAI) treatment. **(a)** and **(b)**, The calculated atherogenic index (AI) and coronary risk index (CRI) increased significantly in the CKD placebo group. All the risks for

the heart were reduced upon T3 and BAI treatment, with the maximum protective effect observed with the combined dose. **(c)** Histopathological analysis of kidney sections with hematoxylin and eosin (H&E) staining indicating myocardial damage in CKD patients. **(d)** Represents the calculated fibrotic area in the heart section via ImageJ. ∗, ∗∗, and ∗∗∗ indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Fig. 11. Schematic representation of bidirectional treatment using triiodothyronine and baicalein to upregulate and stabilize Klotho expression.

Table legends:

Table 1. Mouse nucleotide sequences of the primers used for qRT–PCR analysis

Table 2. Kidney function test: Serum and urine biochemical parameters

Table 3. Evaluation of the degree of damage in renal histological sections

Table 4. Serum parameters for anemia

Table 5. Effects of T3, BAI, and the combined treatment (T3 + BAI) on the serum parameters of minerals and Bone disorders