# A Dual intervention of Triiodothyronine and Baicalein bi-directionally upregulates Klotho with attenuation of chronic kidney disease and its complications in aged BALB/c mice

## Authors

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## Abstract

Chronic kidney disease (CKD) presents a pressing global health challenge, characterized by progressive renal function decline and heightened morbidity and mortality. The interplay between CKD and hypothyroidism, particularly through the non-thyroidal low triiodothyronine (T3) syndrome, exacerbates disease progression and elevates mortality rates. Perturbations in the hypothalamic-pituitary-thyroidal (HPT) axis contribute to this scenario, while fibrotic kidneys exhibit diminished levels of the protective protein Klotho due to abnormal activation of the Wnt/β-catenin pathway. Leveraging our previous findings showcasing T3's ability to downregulate aberrant Wnt/β-catenin pathway activity by enhancing Klotho expression, both in vitro and in vivo, and Baicalein's direct inhibition of the Wnt pathway in C. elegans, we investigated Klotho's potential as a molecular link between CKD and hypothyroidism. Through experiments utilizing an adenine-induced CKD aged mouse model, we aimed to enhance Klotho expression via exogenous T3 administration and bidirectional Wnt pathway blockade using Baicalein (BAI). Our results demonstrate 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, targeting Klotho biology directly enhanced by T3 and sustained by T3 coupled with Wnt pathway blockade using BAI, presents a promising strategy for CKD management. Particularly relevant for elderly CKD patients with hypothyroidism, this approach holds potential to ameliorate renal degradation. Our findings underscore the substantial therapeutic promise 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 an abnormal rise 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]. A reduced expression of both triiodothyronine (T3) and thyroxine (T4) with elevated levels of thyroid-stimulating hormone (TSH) was commonly observed in CKD due to dysregulation of the hypothalamus-pituitary-thyroid (HPT) axis [7]. Additionally, a reduction in glomerular filtration rate (GFR) level impairs the conversion of T4 to T3 through uraemia. This may lead to increased mortality in uraemic patients [8]. The suppression of T3 in uraemia 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 choroid plexus in the brain. 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 N-terminus, a single transmembrane domain at C-terminus, and a short cytoplasmic domain (10 amino acids) [11,12]. Membrane Klotho is proteolytically cleaved and shed by different types of secretase like α, β, and γ secretase [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 form of Klotho were 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. A notable observation from our prior studies revealed that T3, a thyroid hormone, could enhance 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.

Simultaneously, the role of the evolutionarily conserved Wnt/β-Catenin pathway in the pathogenesis of CKD is also well established. Recent observations suggest that there was aberrant activation of the Wnt/ β-Catenin pathway in many kinds of kidney disease, including CKD [20,21]. Wnt proteins secret extracellular signal molecules that play an essential role in the formation of the nephron and renal development [22]. Wnt proteins interact with their receptors and co-receptors and induce downstream signaling events, which leads to the stabilisation of β-catenin. Excess of stabilised 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 expressions [26]. On the contrary, Klotho could 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, we have also identified a clue from our previous research indicating that T3 can notably augment various forms of kidney-specific Klotho protein while reducing the elevated 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*, which possesses many beneficial properties, including anti-inflammation, antioxidative, antimicrobial, and immuno-regulatory. It has a time-honoured place in Asian traditional medicine for treating liver, kidney, and cardiovascular diseases [32]. It is reported that BAI could protect against different types of carcinoma and nephrotoxicity by inhibiting the Wnt/β-catenin pathway [33–35]. Preliminary studies from our lab using nematode *C. elegans* revealed that BAI could enhance Klotho expression by inhibiting Wnt ligands [30].

This study proposes a novel bidirectional targeting approach, leveraging both exogenous hormone (T3) and the natural compound BAI, to achieve a 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 beckons 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 experiments were conducted as per the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi, India under the supervision of IAEC. Animals were housed in conventional conditions: $23^0 \pm 2^0$ C, 12:12 light and dark cycle, standard diet, and water *ad libitum*. 20-months old Balb/c mice were used for this study. After 15 days of acclimatisation, animals were grouped, with six animals in each group based on their weight. Blood collection and euthanasia of 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 50 mg/kg b.w. of adenine (Sigma Aldrich, USA) dissolved in 0.5% carboxy methyl cellulose (CMC) via oral dose for 21 days, while the control animal received 0.5% CMC only for 21 days. After CKD induction, the animals were further divided into different groups. The CKD group received a vehicle for T3 and BAI (0.1N NaOH and DMSO) and was named the Placebo group. 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) were named after their treatment. The T3 and BAI dose was fixed based on 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 both (T3 + BAI group). BAI was administered for five days consecutively after 21 days, whereas T3 was administered in the last three days, i.e., the $24^{th}$-$26^{th}$ day. All the animal experiments used in the manuscript follow the recommendation in 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, haemoglobin, total cholesterol (TC) total triglyceride (TG), HDL-C, ALP-activity, bilirubin and Urine creatinine, urea has been measured using Agappe diagnostic kit (Agappe diagnostic, India) as per manufacture's instruction. LDL-C and VLDL-C were calculated using 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 were measured by competitive ELISA and TSH by sandwich ELISA method as per the manufacturer's instruction.

**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. The kidney damage was evaluated based on the parameters like inflammatory infiltration, protein cast, interstitial fibrosis, and ECM deposition. The parameters were measured based on ten randomly selected fields from each section under x400 magnification. All the parameters were measured using Image J software.

**RNA isolation and qRT-PCR analysis**

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

**Western blotting analysis**

For western blot analysis, all reagents were purchased from Sigma Aldrich, USA, unless mentioned. Kidney tissue was homogenised in RIPA lysis buffer with protease and phosphatase inhibitors. An equal concentration of proteins was loaded and electrophoresed on a 10% gradient tris-glycine gel. Proteins were electrophoretically transferred to an immobile-P PVDF membrane (Roche, USA). After blocking with 5% non-fat skimmed milk for 1 hr at room temperature, the membranes were incubated in primary antibody (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 $4^0$C

overnight. After thorough washing with buffers, the membranes were incubated in a secondary antibody (sc-516102) for 1 hr at room temperature. The bands were then developed in chemiluminescence substrate using the Vilber Chemi Doc imaging system. The intensity of the band was measured using ImageJ software, and relative expression was determined with GAPDH.

**Antioxidant enzymes assay**

**Catalase assay**

Catalase activity was measured as described previously [43]. The tissue homogenate was incubated in the substrate at $37^0$C for three minutes. After the incubation, the reaction was stopped using ammonium molybdate. The absorbance of the molybdate and hydrogen peroxide yellow complex was measured at 374nm against blank. Specific activity was expressed as a U/mg of protein.

**SOD activity**

SOD activity in the tissue homogenate was measured by the method of Kostyuk V A *et al* [44]. SOD activity was measured by the inhibition of auto-oxidation of quercetin by the enzyme at pH 10. The result was expressed as U/mg of protein.

**Statistical analysis**

A two-way analysis of variance with post hoc Tukey correction was used to compare the means of different groups. All experiments were performed in triplicates and reported as mean ± SEM. The graphs were plotted using GraphPad Prism software (GraphPad Software, San Diego, CA, USA), and statistical significance was also measured using the same software. Error bars represent SEM. P values < 0.05 were considered statistically significant.

## Results:

**CKD reduces body weight and improves with bidirectional treatment**

The body weight of all animals was measured every two days during the treatment period. It was observed that the body weight of animals significantly decreased during the adenine administration period. At the adenine diet endpoint (21st day), the adenine-fed placebo group animals weighed 31% lower than the control group ($p<0.001$). On the contrary, when the adenine-fed animals were treated with either T3, BAI, and combined dose (T3 + BAI) separately, significant improvements in body weight at the end of treatments (27th day) were observed. Where T3 treated animals showed an increment in body weight of 17.9% ($p<0.001$),

BAI treated with 16.6% ($p<0.001$), and the combined (T3 + BAI) treated animals exhibited the highest gains with an increment of 19.2% ($p<0.001$) from that of 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 using serum and biochemical kidney injury markers. Nm, We initially observed that serum creatinine, urea, and BUN concentrations were significantly higher in the placebo group than in the control group. The percentage of increase for creatinine was 8.8-fold ($p<0.001$), Urea 1.56-fold ($p<0.05$), and BUN with 1.56-fold ($p<0.05$). On the contrary, the placebo group's urine creatinine and urea concentrations were significantly lower than the control group, with 3.12-fold ($p<0.01$), and 2.08-fold ($p<0.01$), respectively. Moreover, the urine albumin 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 in combination (T3 + BAI) ameliorates the changes observed in the placebo group towards the normal range. Specifically, in comparison to the treated groups, the combined (T3 + BAI) treatment produces a more pronounced effect wherein serum creatinine, urea and BUN declined by 3.8-fold ($p<0.01$), 1.63-fold, ($p<0.01$), and 1.68-fold ($p<0.001$), respectively from the placebo group. **(Table 2)**

**Effect of T3, BAI, and combined (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 check for pathological renal conditions **(Fig. 3).** H & E staining **(Fig. 3a)** revealed that the CKD mice (Placebo group) exhibited significant monocyte and lymphocyte infiltration (A), protein cast (B), and cytoplasmic vacuolation (C) in the 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 either T3, BAI or in combination (T3 + BAI) significantly improved the damages. The damages in the renal sections were evaluated based on ten randomly selected fields per section, which were examined under x400 magnification. The evaluation criteria and observed results are presented in **Table 3**. Overall, our results showed that the combined treatment could produce 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 experimentally adenine-induced CKD, we found that the mRNA expression of M-Klotho and Sp-Klotho decreased significantly in the placebo group in comparison to the control group by 3.25-fold ($p<0.001$) and 2.35-fold ($p<0.001$) respectively **(Fig. 4a, c).** On the contrary, the M-Klotho expression increased significantly upon T3, BAI, and combined (T3 + BAI) treatment by 4.5-fold ($p<0.001$), 3.6-fold ($p<0.01$), and 5.5-fold ($p<0.001$), respectively, from 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 reduces remarkably in placebo mice by 91.6% and 15.5% respectively from the control group **(Fig. 4e, f, g)**. Upon treatment with T3 and BAI, significant enhancement in Klotho protein expression was observed and the combined treatment (T3 + BAI) exhibited higher inducibility in Klotho protein expression than either of the individual treatments.

**Aberrant increase in GSK-3β expression during CKD suppresses maximally 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 checked for the GSK-3β expression, which tends to overexpress in different types of kidney injury. We found a significant increase of 3.3-fold ($p<0.001$) in GSK-3β protein expression in the kidney of the adenine-fed placebo group in comparison to the control group. Interestingly, T3 and BAI treatments counteract the observed expression by a reduction of 2.1-fold ($p<0.001$) and 1.8-fold ($p<0.01$) receptively as compared to the placebo group. Optimum downregulation of GSK3β with a decline of 4.6-fold ($p<0.001$) was observed in the combined (T3 + BAI) treated group **(Fig. 5a, b)**.

**Combined (T3 + BAI) suppresses canonical Wnt/β-Catenin pathway in CKD animals**

Understanding the crosstalk of evolutionarily conserved Wnt/β-Catenin pathway with other signaling pathways is crucial in understanding kidney disease progression and in elucidating the therapeutic potential of treatment protocols [49]. To check for the effect of our treatments on the canonical Wnt/β-Catenin pathway, the mRNA expression of Wnt ligands like Wnt1, 3, 8A, 8B, 10A, and protein expression of β- Catenin and Casein kinase 1 (CK-1) was performed. We found that the mRNA expressions of all Wnt ligands increased significantly in the placebo mice than in the control mice **(Fig. 6a).** However, treatment with BAI and combined (T3 + BAI) represses these mRNA expressions in comparison to the placebo group **(Fig. 6b).** On the

contrary, no significant ($p<0.99$) changes were observed in the group that received only 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 declined by 38.5% ($p<0.01$) in the placebo group than the control group. On the contrary, after T3 and BAI treatments, the protein expressions 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% ($p<0.01$)] has increased significantly towards a normal level. Moreover, the combined treatment group exhibited a maximum decline in β-catenin [58.3% ($p<0.01$)] with an increase of CK-1 [71% ($p<0.01$)] protein expression from 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 recognised 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 mice compared to the control mice **(Fig. 7a).** On treatment with either T3 and/or BAI, a significant reduction in TGF-β mRNA expression by 2.3-fold ($p<0.05$) and 2.6-fold ($p<0.05$) was observed when compared to the placebo group. In comparison, the combined (T3 + BAI) group animals showed a higher reduction of TGF-β mRNA expression by 3.56-fold ($p<0.05$) from that of the placebo group **(Fig. 7b).**

**Protective effect against CKD-induced inflammation**

A pro-inflammatory state is also linked to chronic kidney disease, and persistent low-grade inflammation is increasingly seen to be a defining feature of CKD [51,52]. Increased production and decreased clearance of proinflammatory cytokines contribute to 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.

It was found that protein expression levels of inflammatory kidney markers like nuclear factor-κB (NF-κB) and interleukin 6 (IL-6) increased significantly in the placebo mice compared to the control mice. This includes an increase of NF-κB by 2.7-fold ($p\leq0.001$) and IL-6 by 3-fold ($p\leq0.001$), respectively. Moreover, the T3 and BAI group mice showed significantly lower inflammatory markers than the placebo group. At the same time, individual T3 and BAI treatments significantly reduced N-FκB expression by 2.2-fold ($p\leq0.01$) and 1.8-fold ($p\leq0.01$), respectively, from the placebo group. Similarly, 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, from the placebo group. Maximum reduction in inflammatory markers was observed in the combined treatment group wherein NF-κB expression was reduced by 4.3-fold ($p \leq 0.001$) and IL-6 expression by 3-fold ($p \leq 0.001$) from the placebo mice **(Fig. 8a, b, c).** These findings provide evidence that the combined T3 + BAI could alter an increase in inflammatory cytokines during CKD thereby, could protect the kidneys from further deterioration.

**T3 but not BAI could protect CKD animals from anaemia**

A decrease in one or more important red blood cells parameters, such as the hematocrit, haemoglobin concentration, or red blood cell count, is referred to as anaemia. Since anaemia is a frequent side effect of CKD, it is unquestionably an independent risk factor for worse outcomes in CKD. Anaemia 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 checking the anaemia status in CKD mice and the effect of treatment on it. It was found that the haematocrit percentage and haemoglobin concentration reduced significantly in the placebo group by 27.4% ($p \leq 0.05$) and 23.3% ($p \leq 0.05$), respectively, as compared to the control. An enhanced haematocrit percentage of 17.08% ($p \leq 0.05$) and 15.7% ($p \leq 0.05$), respectively, was observed in the T3 and combined (T3 + BAI) treated group compared to the placebo group. Similarly, haemoglobin concentration increased by 14.7% ($p \leq 0.05$) and 14.7% ($p \leq 0.05$). On the contrary, 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 anaemia (**Table 4)**.

**T3 but not BAI modulates mineral bone disorders in CKD**

CKD-induced mineral bone disorders comprise abnormalities in bone and mineral composition [57]. CKD patients develop an inadequate 1,25 dihydroxy-vitamin 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, serum alkaline phosphatase (ALP) increases in CKD patients [58–60]. Hence, serum phosphate, calcium, Vitamin D3, and ALP activity were estimated to investigate the potential of treatments in modulating mineral and bone disorders. Our results showed a significant increase in the serum phosphate and a decrease in calcium concentration in the placebo mice than 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 brought by the combined treatment. Likewise, an increase in mRNA expression of phosphate channel NPT2a, followed

by a decreased expression of calcium channel TRPV5, was found in CKD animals. However, the combined treatment has a much more positive effect on the altered changes caused by CKD, with reverses the abnormality through decreased NPT2a and increased TRPV5 mRNA expression **(Figure 9)**. A significant decline in serum 1,25, Hydroxy Vitamin D3 level, and an increase in ALP activity have also been observed in the placebo mice compared to control 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]. Heightened proinflammatory microenvironment and other modifying factors (Dyslipidaemia) during CKD predispose individuals to cardiovascular events **(Supplementary Result S3)**. The atherogenic index (AI) and coronary risk index (CRI), calculated based on serum triglyceride, total cholesterol (TC) and HDL-C levels (**Supplementary Table 1)**, showed a significant risk ($p \leq 0.001$) in the placebo group than in the control group **(Figure 10a, b)**. Risk levels pertaining to AI and CRI were reduced in T3 and BAI-treated animals compared to the placebo group. Combined treatment fares better in lowering the AI and CRI predisposition than either of the individual treatment.

Like kidney tissue, the heart tissue has also been checked for damage by H & E staining of thin sections. We found that in the placebo group, there was a significant increase in fibrosis and inflammatory infiltration than in the control mice. Surprisingly, individual T3 and BAI treatments reduced fibrosis, and T3 has a more anti-fibrotic effect than BAI. Moreover, the combined treatment (T3 + BAI) produced a significantly pronounced anti-fibrotic effect than either of the individual treatments **(Figure 10c, d)**. Like 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 mice ($p \leq 0.001$). On the contrary, T3 and BAI could alleviate the activity of both enzymes, and the combined treatment (T3 + BAI) produced the highest activity of both enzymes compared to that of the placebo group (**Table 25)**.

## Discussion:

In this present study, we intend to decipher the protective effect of combined T3 and BAI treatment on adenine-induced old CKD mice. As per our previous findings [19,62], we intend to target CKD bidirectionally using thyroid hormone T3 and BAI. T3 was used with the intention of maintaining a normal HPT axis in CKD, whereas BAI to counteract the exaggerated upregulation of the Wnt/β-catenin pathway, as reported. Interestingly we found that both treatments could enhance Klotho via the different targets. The details of bidirectional treatment have been illustrated in **Figure 11.** Furthermore, as CKD predisposes individuals to other complications like anaemia, dyslipidemia, cardiovascular disease, and mineral bone disorders, we intend to evaluate the therapeutic potential of the individual (either T3 or BAI) versus combined (T3 + BAI) treatment against 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 per Rahman *et al.* 2018 [64]. The successful induction of CKD was confirmed with a significant increase in serum creatinine along with a drastic reduction in the body weight of treated animals. Following CKD induction, 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 enhanced body weight, which provides the first clue as to how much the overall health parameters of CKD mice have improved. In contrast, the animals who received the combination therapy had a more significant gain in body weight. According to some reports, adenine-induced CKD in mice resulted in skeletal and cortical bone loss, especially in males [65]. This implies that our treatments could counteract these damages, thereby with visible and remarkable improvement in their body weight.

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

Previously, researchers have observed that CKD has huge impacts on the HPT axis, resulting in impaired thyroid hormone metabolism. Likewise, subclinical hypothyroidism's prevalence increases with a decrease in the GFR [5,68,69]. Moreover, the prevalence of subclinical

hypothyroidism increases with aging (In aged individual of age 60 or above) which is correlate with the decrease GFR with age [70–72]. Most commonly observed in CKD patients is a decline in serum T3 and T4 levels and an increased 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 using a dose as reported earlier [36,37]. Mice received exogenous T3 to keep their HPT axis from becoming dysfunctional. After 12 hrs of T3 treatment, significantly lower TSH levels were seen only in the T3 and T3 + BAI groups. On the contrary, no significant changes in thyroid profile were observed in only 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 kidney tissue's inability to repair after long-term, chronic damage [73]. Renal cells are replaced by ECM in the glomeruli and interstitium as CKD progresses, which is seen in 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 cast was observed in the CKD kidney. Similarly, there was enhanced ECM deposition, characterized by Masson's trichrome staining after the adenine dose. Even if T3 and BAI therapy could reduce the damage, the rate of improvement was modest. In contrast, a higher rate of matrix remodelling was seen in the combination treatment group, as evidenced by a considerable decrease in fibrotic area and ECM deposition. These findings indicate that combination (T3 + BAI) therapy in CKD mice has a more significant effect in preventing the kidney from further deterioration from CKD.

The expression of Klotho is 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 decline 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 declines in CKD kidneys. A similar result was found in 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 towards the normal range after T3 and BAI treatment but not higher than that of combination treatment. As reported previously, we speculate that BAI's observed increased

levels of Klotho may be exerted via its inhibition of the Wnt/β-catenin pathway, whereas T3 may directly activate Klotho, as evidenced from our previous report.

Furthermore, it is widely established that 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 TGF-1, GSK3β's significance in the profibrogenic plasticity of renal tubular cells in 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, it is crucial to ascertain if GSK3β expression changes after treatments. 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, the combination therapy had the most significant impact. These findings imply that protection from tubular damage can be attained by raising the Klotho level bidirectionally.

The canonical Wnt/β-catenin pathway, as the evolutionarily conserved signaling pathway, controls the pathogenesis of many diseases. Overexpression of this pathway has been observed in different kinds of kidney injury [89–92]. The sustained activation of the canonical Wnt/β-catenin pathway drives acute kidney injury (AKI) to CKD progression [93]. Similarly, we observed that the adenine-fed animal had more robust activation of the Wnt/β-catenin pathway, which was demonstrated by increased mRNA expressions of the different Wnt ligands and decreased protein expression of CK-1 and increased production of β-catenin protein in the kidney. The natural compound BAI effectively reduced the increased mRNA expressions of all the Wnt ligands in CKD mice. Additionally, T3 and BAI were effective in reversing the impaired expression of β-catenin and CK-1 in CKD mice, with the combination therapy leading to a greater level of β-catenin degradation. We can suggest that the effect of T3 on Wnt inhibition may be due to its impact on increased Klotho expression, as Klotho could inhibit the Wnt/β-catenin pathway without altering the Wnt mRNA expression [94–96]. Likewise, 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 levels of Klotho protein will be 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 developing 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 clinical trials based 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 lessen structural renal injury and renal fibrosis in CKD [101,102]. In another finding in renal cultured epithelial cells, 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]. As per our current finding, adenine-induced CKD mice had higher TGF-β mRNA expression. This overexpression correlates with upregulation of the Wnt/β-catenin pathway, culminating in decreased Klotho expression. However, a significant decline in the level of TGF-β was observed in the combination treatment. Our findings corroborate with reports elsewhere [104] wherein Klotho can inhibit TGF-β signaling, which can be beneficial in suppressing activity against renal fibrosis.

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 an excess of 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 like NF-κB, TNF-α, and interleukins were reported in adenine-induced CKD mice. Similarly, in our investigation, NF-κB and IL-6 protein expression dramatically increased in the kidney of CKD mice. According to our earlier findings, the anti-inflammatory effect 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 can be linked to the combination treatment's considerable enhancement in Klotho expression.

Overproduction of oxidizing free radicals and inadequate anti-oxidant breakdown of these pro-oxidants lead to oxidative stress. Due to reduced anti-oxidants and exponential ROS formation, CKD is a condition with significant oxidative stress [107]. There is a strong correlation between the generation of ROS and Klotho. To combat oxidative stress, Klotho is reported to downregulate the insulin/IGF-1/PI3K signaling, activating the 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 conversation 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 the adenine-induced CKD kidney [112]. Hence, we determined the kidney's anti-oxidant enzymes, like catalase and SOD

activity. As expected, the adenine-induced CKD kidney showed reduced expressions of both the anti-oxidant enzymes. All our treatments could be able to lessen the altered antioxidant enzymes activities (**Supplementary Results S2)**.

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

Through the decreased excretion of inorganic phosphate (Pi), which is brought on by a low level of 1,25 dihydroxy vitamin D, CKD is also linked to mineral and bone diseases. This decreased 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 one of the biomarkers to determine high-turnover bone disease, which usually increases in CKD patients [119]. Likewise, we observed that adenine-induced CKD animals significantly increased serum ALP activity and Pi level, with decreased serum Vit-D3 and $Ca^{2+}$.

A reduction in ALP activity with an increased Vit-D3 level was observed to be produced by T3 treatment only. On the contrary, the 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 is mentioned 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 in their combined treatment. Hence, the phosphate and calcium channel mRNA data corroborates with the serum Pi and $Ca^{2+}$ profiles. The observed effect on NPT2a and TRPV5 is speculated through increased Klotho expression by T3 and BAI.

Chronic kidney disease is a systemic pathology with dyslipidemia commonly observed among CKD patients. As renal function declines, the prevalence of hyperlipidemia increases. A high degree of elevated LDL cholesterol and triglyceride is proportional to the severity of renal impairment [5]. Systematically, beneficial effects of the treatment have also been observed. For instance, in lipid profile parameters, the serum triglyceride, cholesterol, LDL-C, and VLDL-C level increased significantly, with a decrease in HDL-C levels in CKD mice. As observed, the

combined T3 and BAI treatment protects against CKD-induced dyslipidemia (**Supplementary Results S3)**.

Furthermore, dyslipidemia and elevated serum TSH level 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 higher in CKD mice and reduced after T3 and BAI treatment, with the lowest risk observed in animals who received the combined treatment. Additionally, as CKD mice showed a higher risk for CVD, we checked the heart tissue for fibrosis using H & E staining. Surprisingly, the CKD heart also exhibited significant fibrosis. As we observed in the kidney, in heart tissue, the combined treatment fares better in giving protection than individual treatment, as seen with a reduction in the fibrotic area, which is a clear indication of its protective effect from CVD. The increased oxidative stress in the kidney intends us to check for the anti-oxidant enzyme activity in heart tissue. Both catalase and SOD activity were reduced in CKD and increased after treatments indicating the protective effect of Klotho even in the heart tissue.

## Disclosure

All the authors declared no competing interests and have consented to the manuscript for submission

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## Credit authorship contribution statement

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

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

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

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### Data availability

The datasets generated and/or analysed in this study are available from the corresponding author Kitlangki Suchiang on 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 |
|---|---|---|---|---|---|
| **Haematocrit %** | 45.93 ± 0.961 | 35.30 ± 0.6580 # | 41.32 ± 0.607 * | 34.93 ± 0.809 ns | 40.86 ± 0.455 * |
| **Haemoglobin (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.**

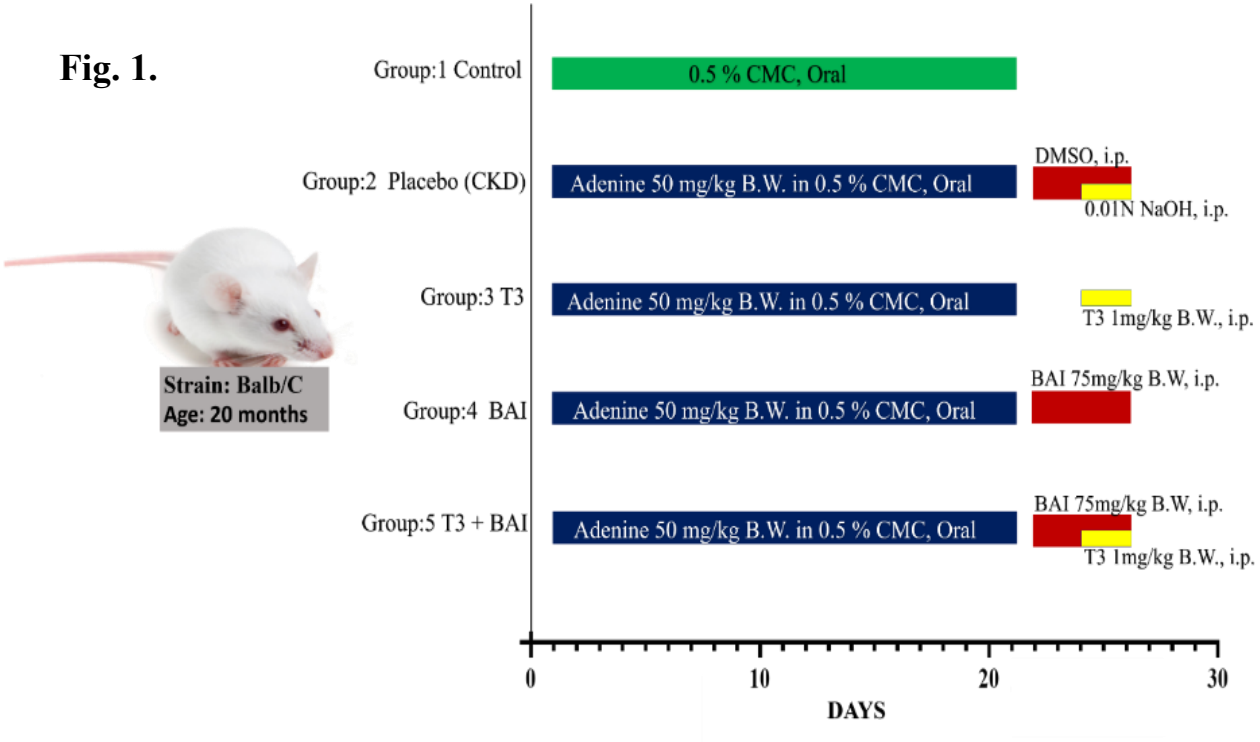



**Fig. 2.**

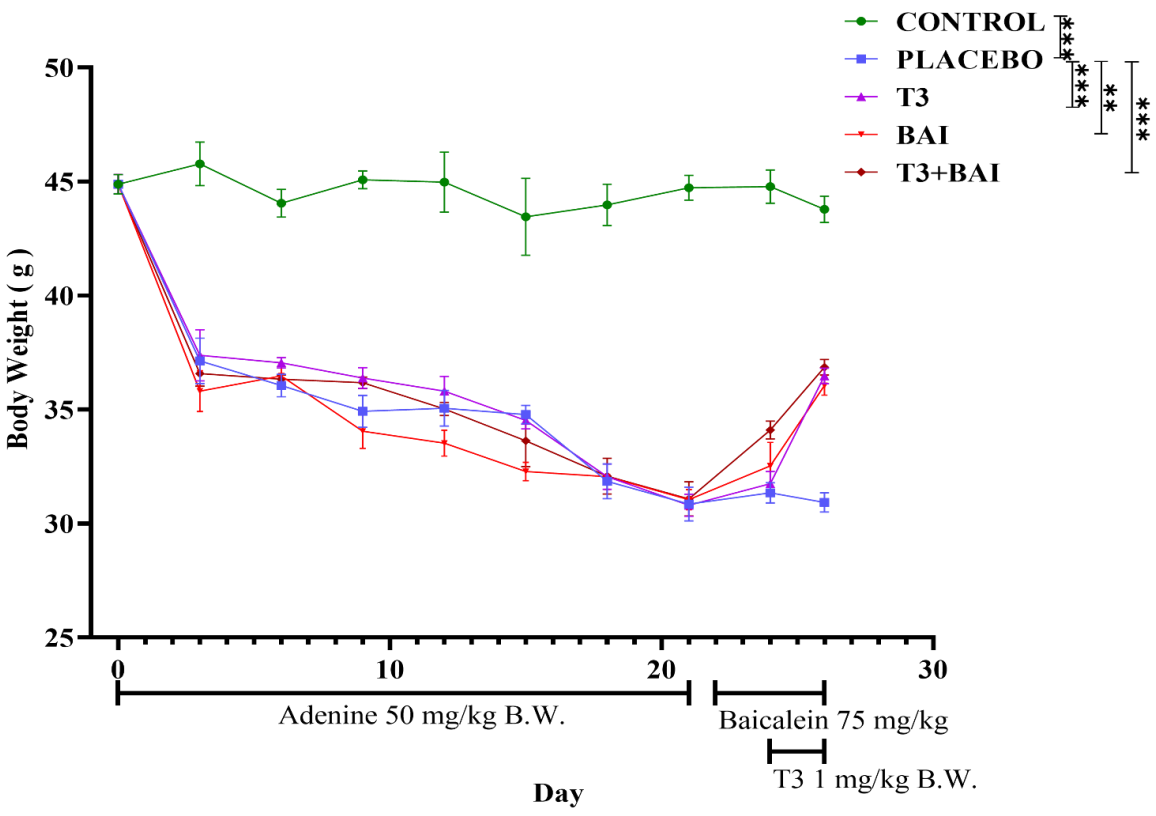


**Fig. 3.**

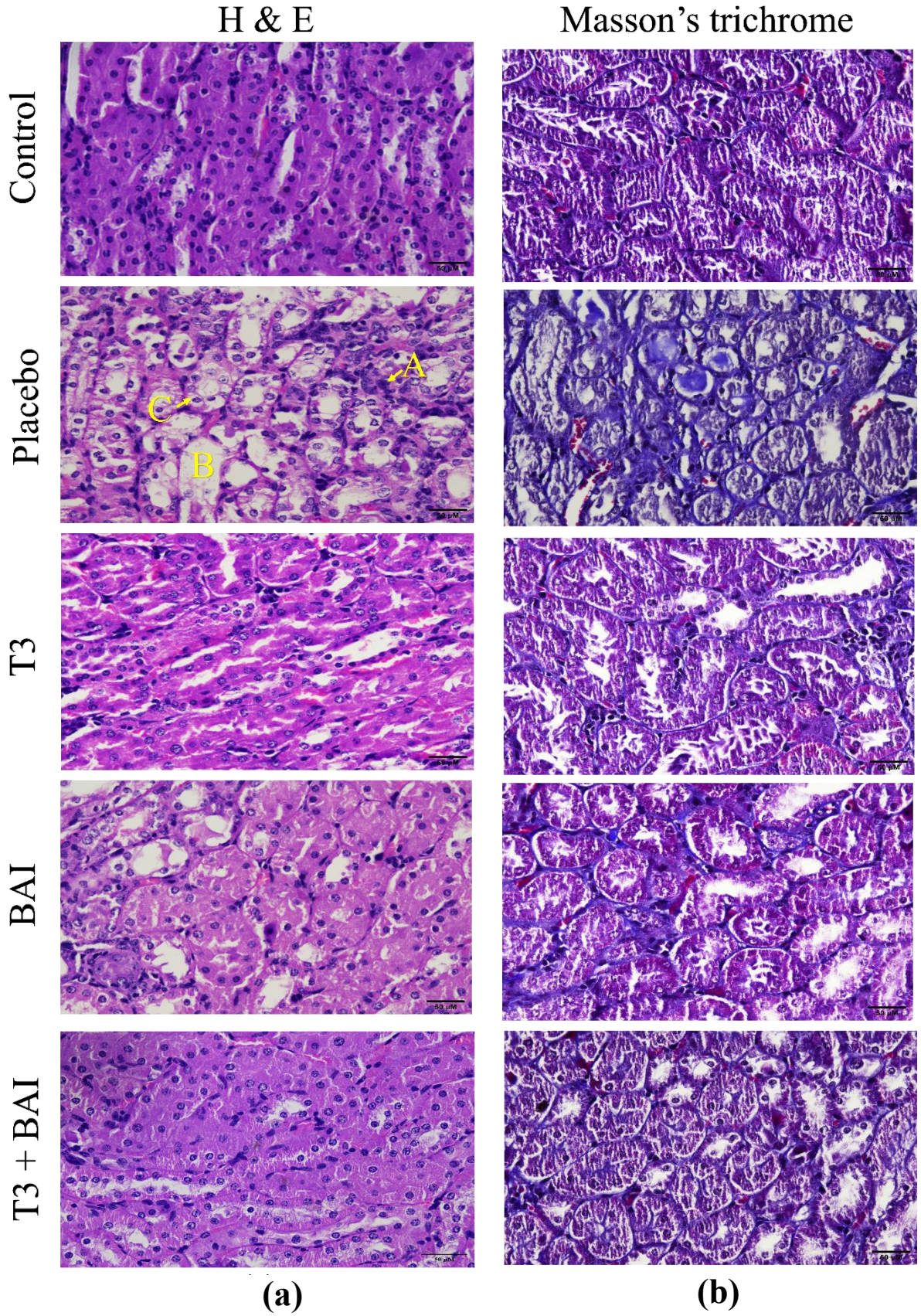
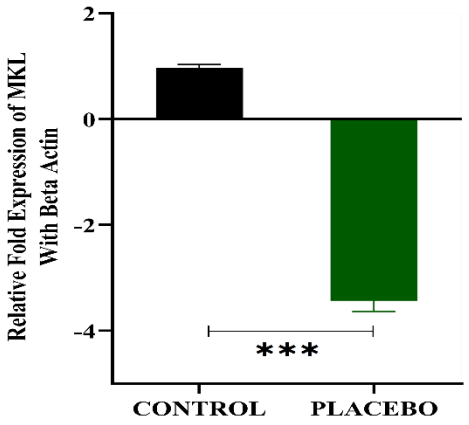
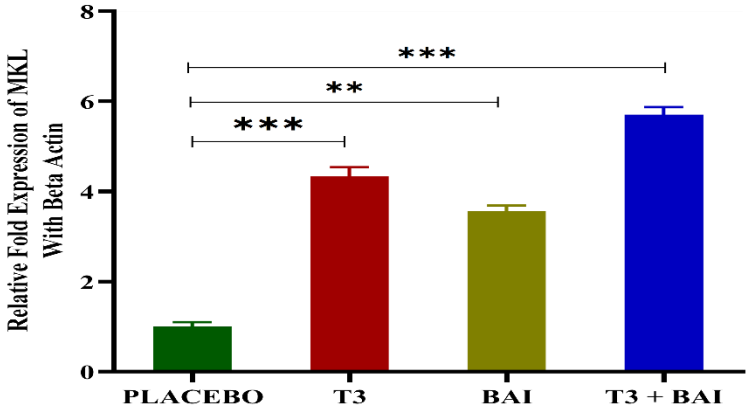


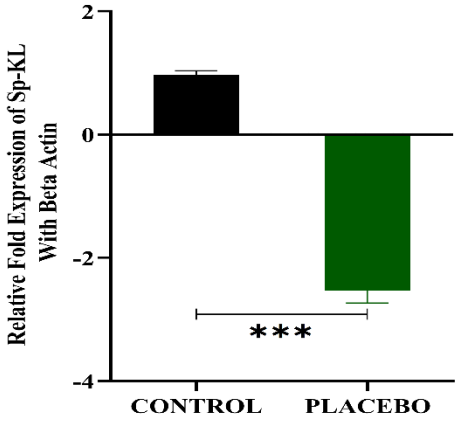
**Fig. 4.**


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

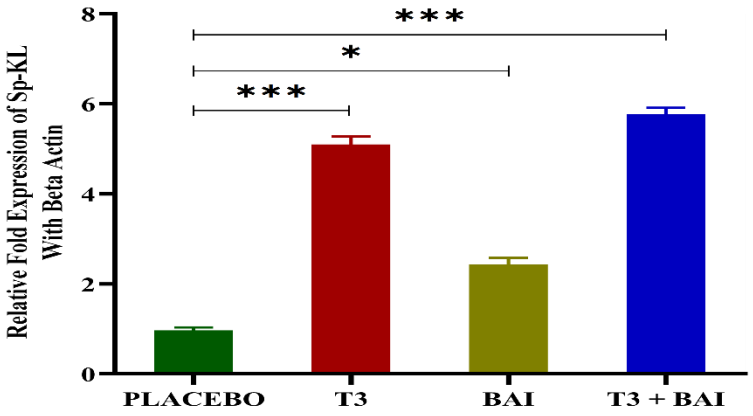
(a)


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

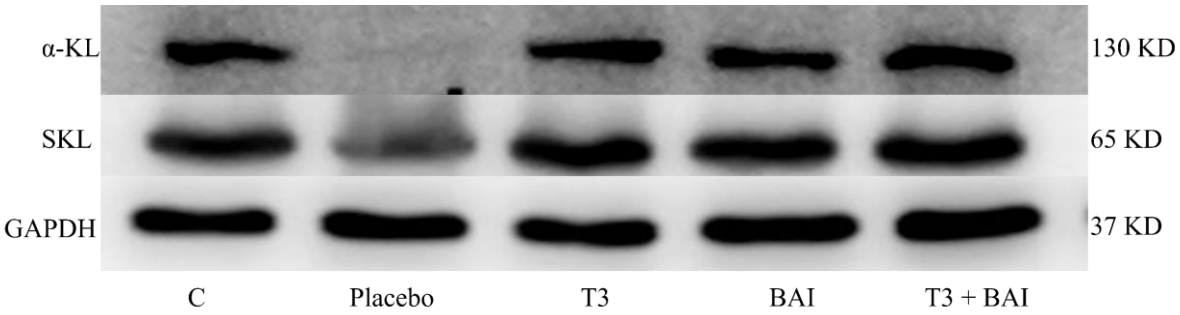
(b)


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

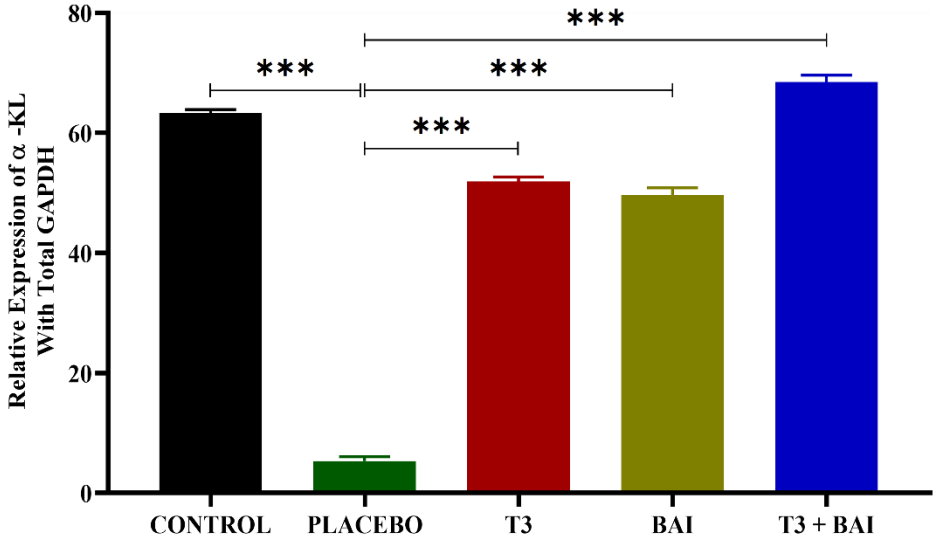
(c)


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

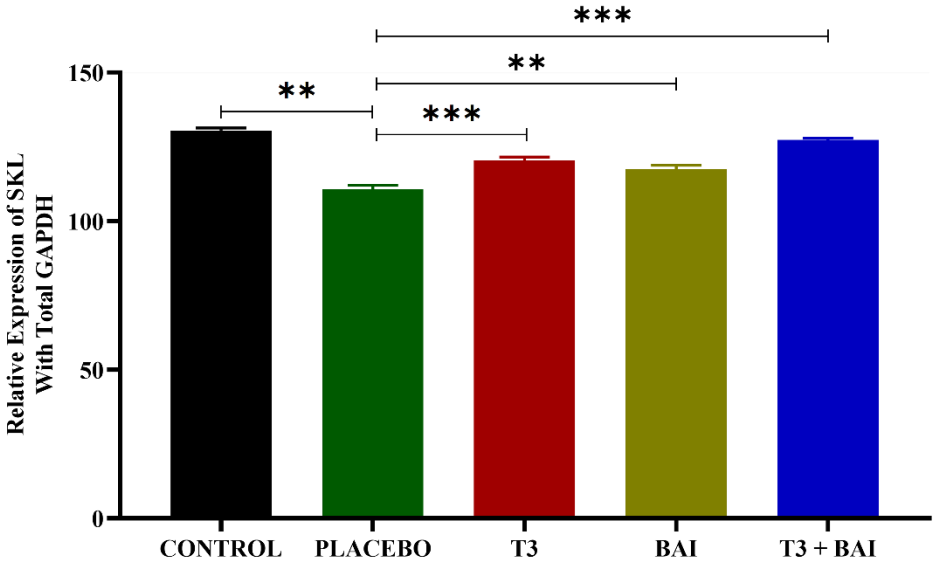
(d)


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

(e)

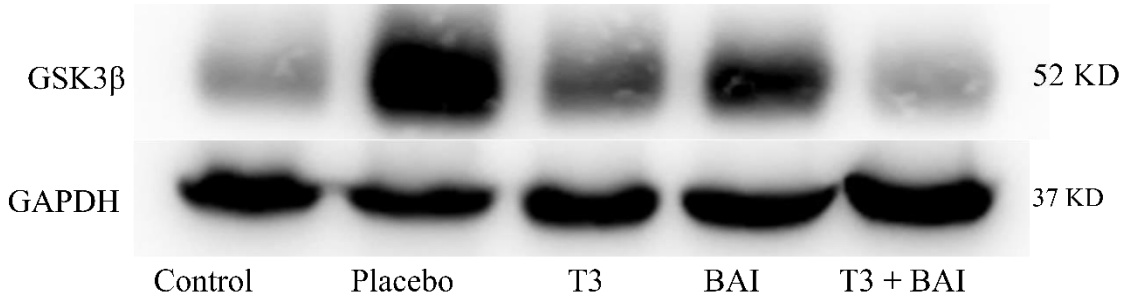

Relative Expression of α -KL
With Total GAPDH
80
60
40
20
0
***
***
***
***
CONTROL
PLACEBO
T3
BAI
T3 + BAI

**(f)**

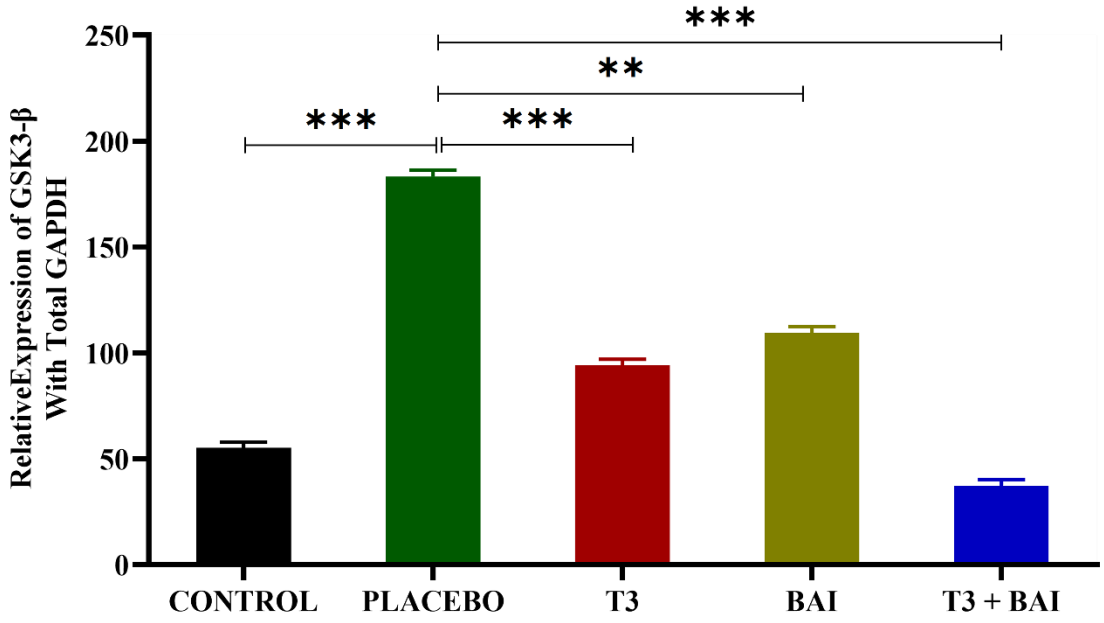

Relative Expression of SKL
With Total GAPDH
150
100
50
0
**
***
**
***
CONTROL
PLACEBO
T3
BAI
T3 + BAI

**(g)**

**Fig. 5.**

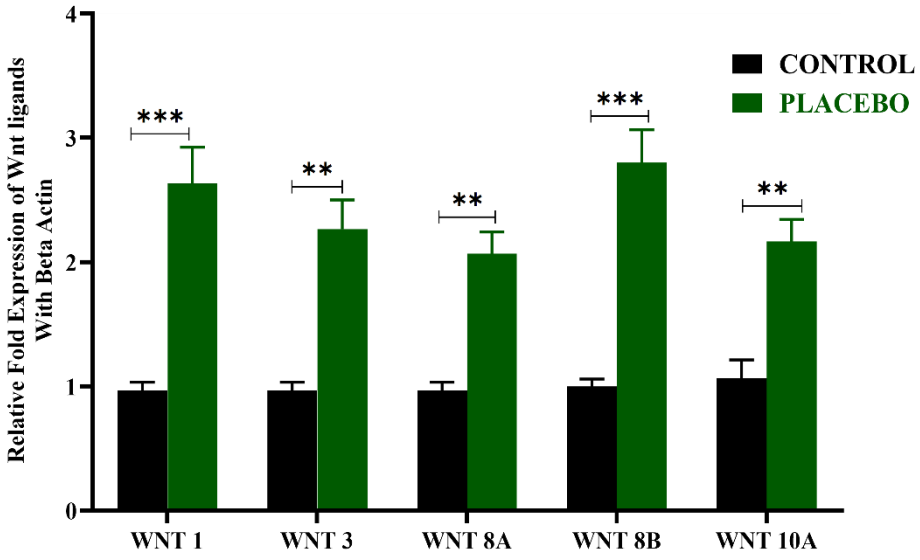

GSK3β
52 KD
GAPDH
37 KD
Control
Placebo
T3
BAI
T3 + BAI

**(a)**

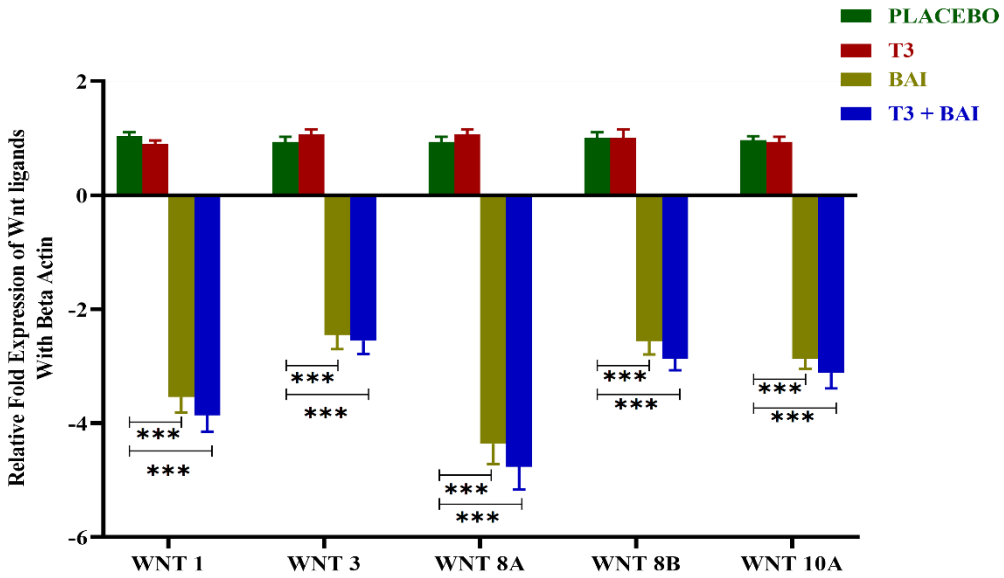

RelativeExpression of GSK3-β
With Total GAPDH
250
200
150
100
50
0
***
**
***
***
CONTROL
PLACEBO
T3
BAI
T3 + BAI

**(b)**

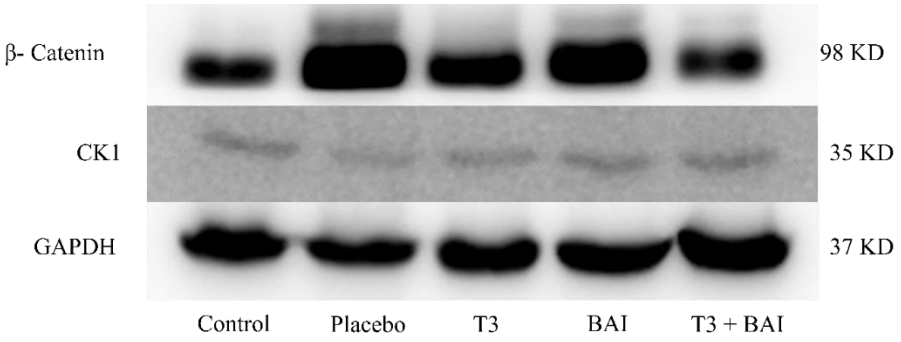
**Fig. 6.**


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

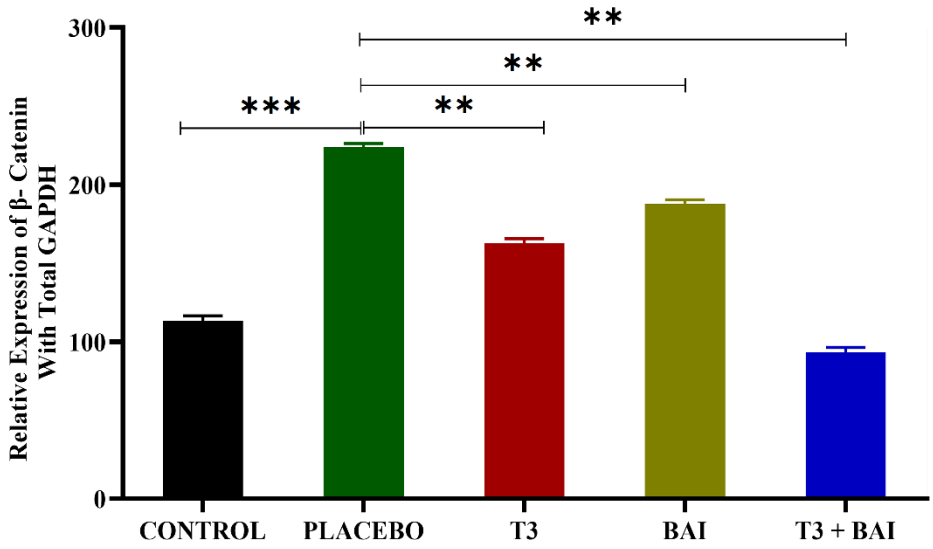
**(a)**


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

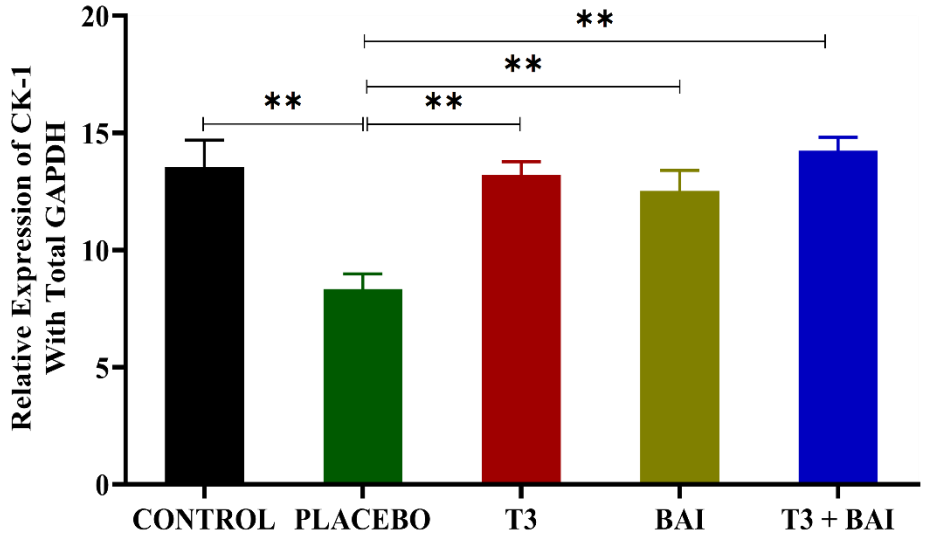
**(b)**


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.**

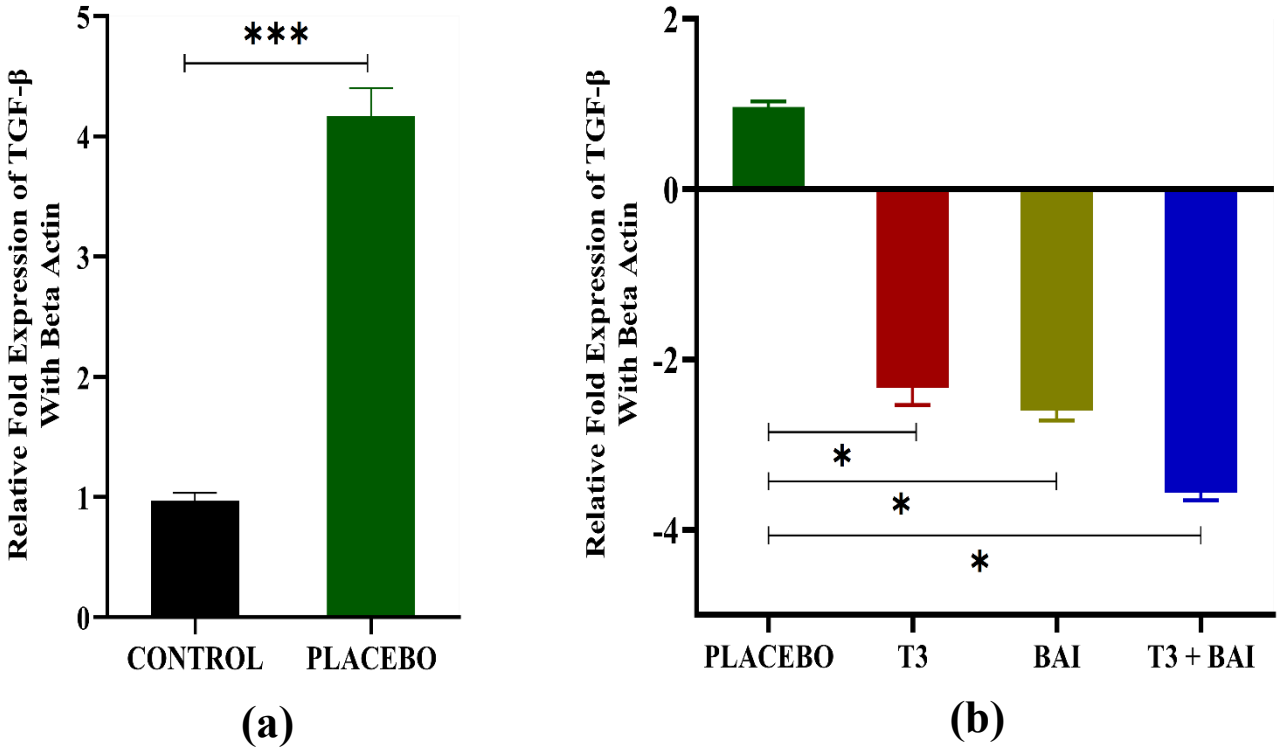

Relative Fold Expression of TGF-β With Beta Actin
5
4
3
2
1
0
***
CONTROL
PLACEBO
(a)
Relative Fold Expression of TGF-β With Beta Actin
2
0
-2
-4
*
*
*
PLACEBO
T3
BAI
T3 + BAI
(b)

**Fig. 8.**

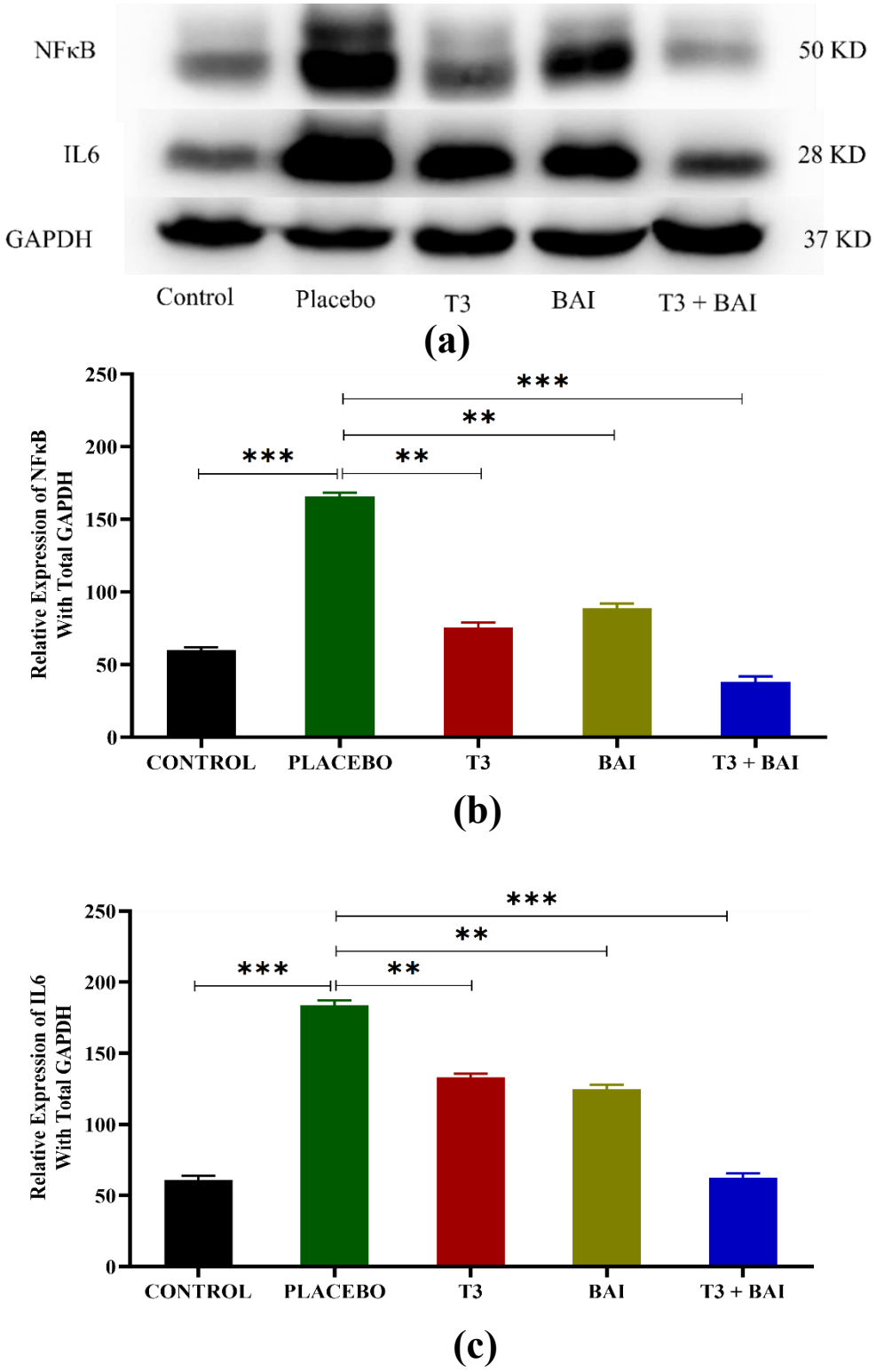

NFκB
IL6
GAPDH
50 KD
28 KD
37 KD
Control
Placebo
T3
BAI
T3 + BAI
(a)
Relative Expression of NFκB With Total GAPDH
250
200
150
100
50
0
***
**
***
**
CONTROL
PLACEBO
T3
BAI
T3 + BAI
(b)
Relative Expression of IL6 With Total GAPDH
250
200
150
100
50
0
***
**
***
**
CONTROL
PLACEBO
T3
BAI
T3 + BAI
(c)

**Fig. 9.**

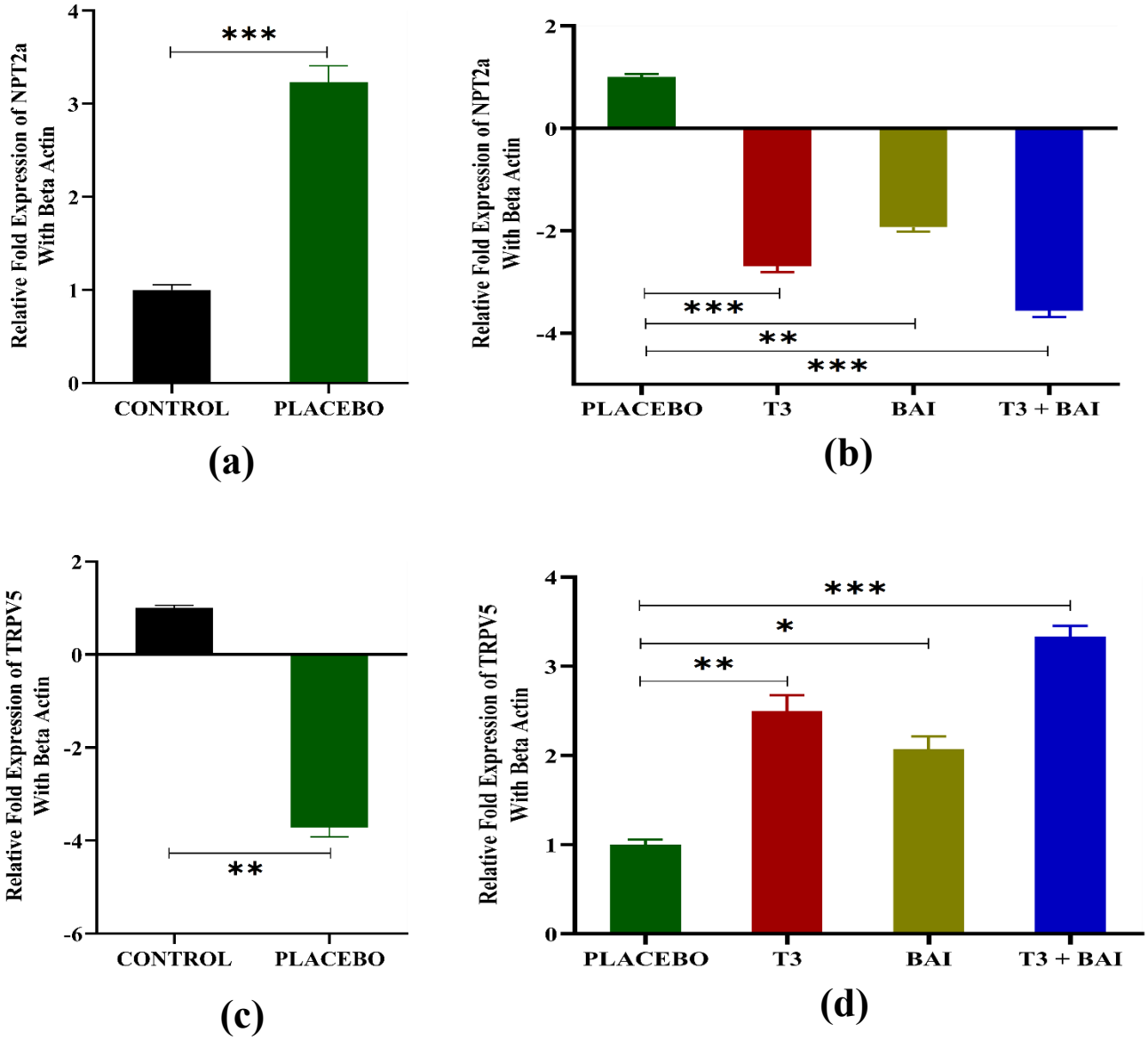

Relative Fold Expression of NPT2a With Beta Actin
***
CONTROL
PLACEBO
(a)
Relative Fold Expression of NPT2a With Beta Actin
***
**
***
PLACEBO
T3
BAI
T3 + BAI
(b)
Relative Fold Expression of TRPV5 With Beta Actin
**
CONTROL
PLACEBO
(c)
Relative Fold Expression of TRPV5 With Beta Actin
***
*
**
PLACEBO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

**Fig. 10.**

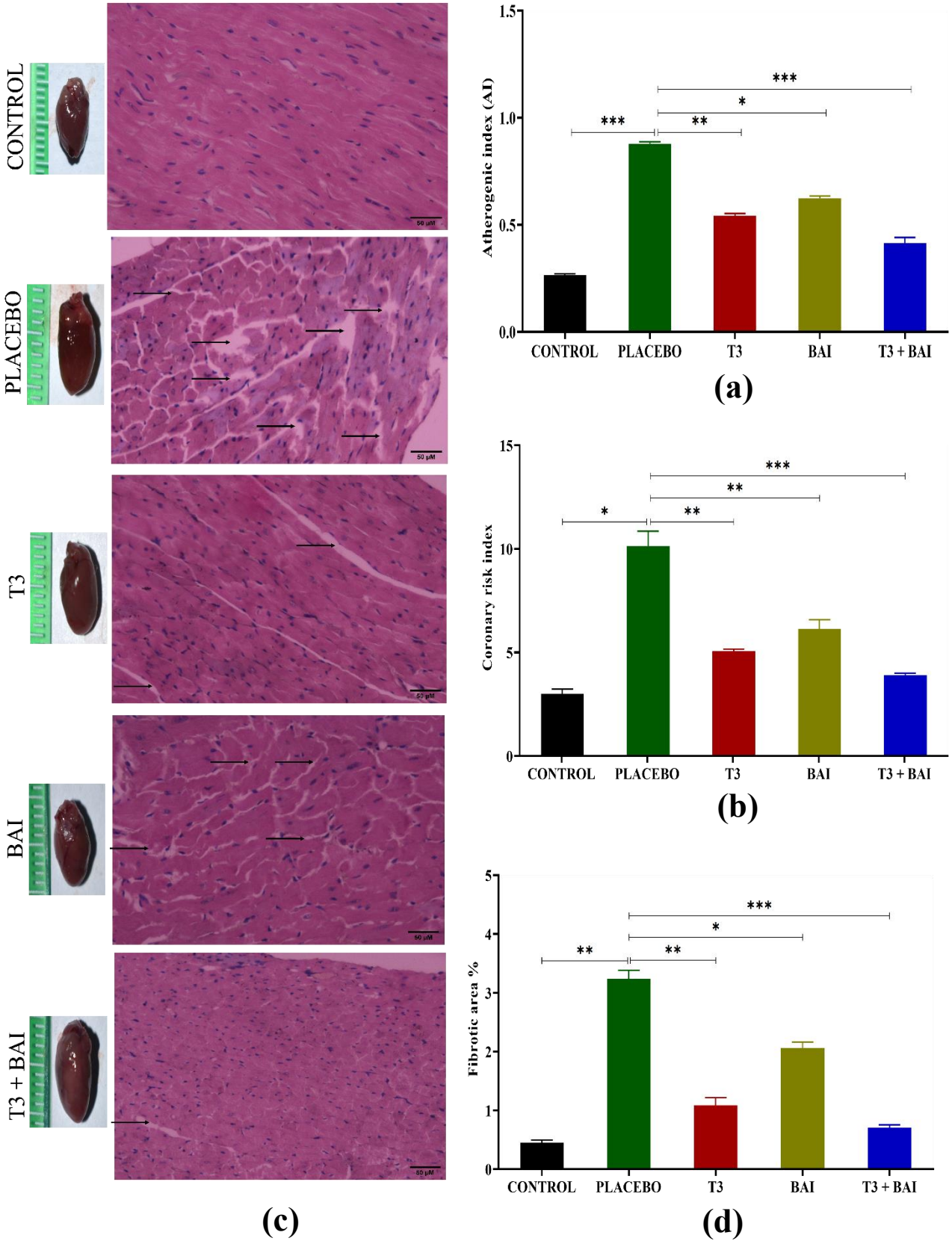

CONTROL
PLACEBO
T3
BAI
T3 + BAI
Atherogenic index (AI)
1.5
1.0
0.5
0.0
***
**
*
CONTROL
PLACEBO
T3
BAI
T3 + BAI
(a)
Coronary risk index
15
10
5
0
(b)
Fibrotic area %
5
4
3
2
1
0
(c)
(d)

**Fig. 11.**

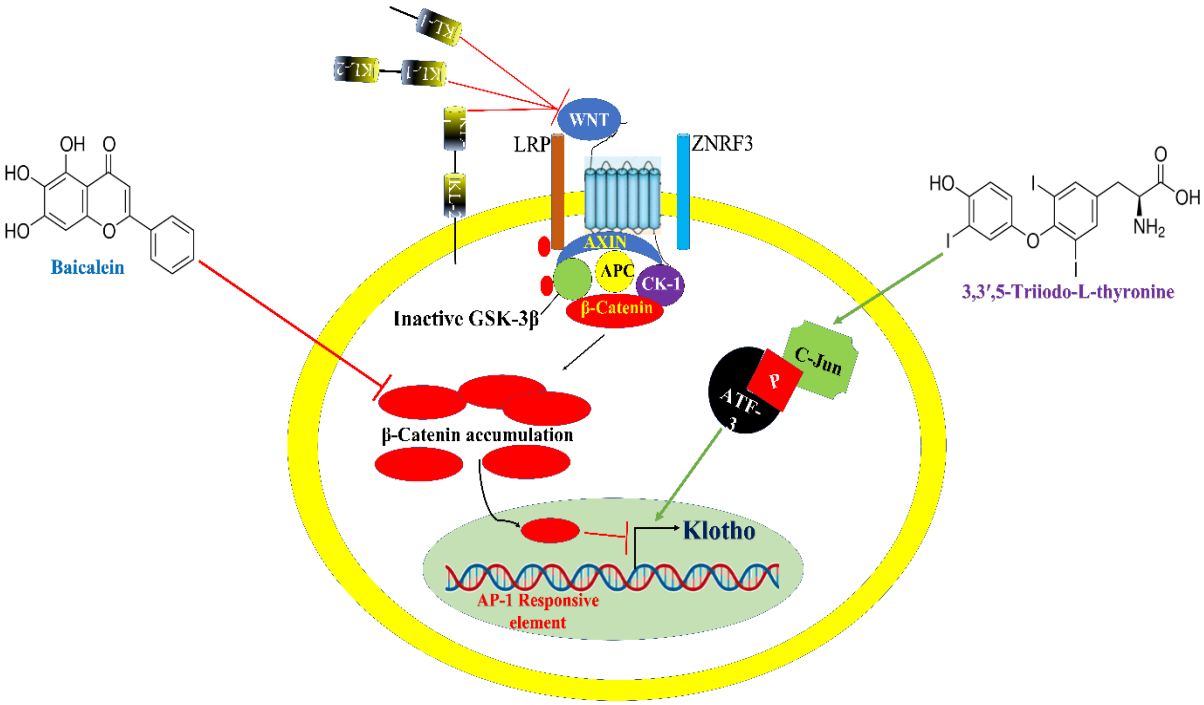



**Figure Legends:**

**Fig. 1. Animal grouping and treatments.**

**Fig. 2. Body weight profile of Balb/C mice during CKD induction and treatments.** Body weight was measured every 2-day interval during the treatment. A significant reduction in body weight has been observed after adenine treatment. However, treatment with eitherT3, Baicalein (BAI) and combined (T3 + BAI) showed a significant gain in the body weight of animals. n = 10 per group. ∗∗, and ∗∗∗ indicate the significance level at $p \leq 0.01$, and $p \leq 0.001$, respectively.

**Fig. 3. Analysis of renal pathological sections in CKD.** Indicating the protective effect of T3, BAI, and their combined treatment (T3 + BAI) against CKD-induced renal fibrosis. **(a)** Hematoxylin and eosin (H & E) staining of renal pathological section (x400), **(b)** Masson's trichrome staining for extracellular matrix deposition analysis (x400). **A, B** and **C** represents inflammatory infiltration, protein cast, and cytoplasmic vacuolation respectively. The scale bar represents 50 µM.

**Fig. 4. Effect of T3, BAI, and combined (T3 + BAI) on Klotho expressions in CKD kidney.** Klotho mRNA, i.e., membrane Klotho (M-KL) and spliced Klotho (Sp-KL) and protein i.e., α-KL and S-KL expression decreased in CKD. After treatment with T3 and BAI in CKD mice, of both mRNA and protein expressions increased significantly, with the combined treatment (T3 + BAI) exhibiting a maximum increase in expressions. **(a)** and **(b)** represents 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)** represent densitometric analysis of α-KL, and

S-KL, respectively. Data are expressed as means ± SEM from 3 independent experiments. ∗, ∗∗, and ∗∗∗ indicate the significance level at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

**Fig. 5. Effect of T3, BAI, and combined (T3 + BAI) on GSK-3β protein expression.** T3 and BAI successfully reduced GSK-3β protein expression, with a higher reduction observed in the combined (T3 + BAI) treatment. **(a)** and **(b)** represents western blot analysis of GSK-3β and its densitometric analysis in CKD mice. Data are expressed as means ± SEM from 3 independent experiments. ∗∗, and ∗∗∗ indicate the significance level at $p \leq 0.01$, and $p \leq 0.001$, respectively.

**Fig. 6. Effect of T3, BAI, and combined (T3 + BAI) on Wnt β-Catenin Pathway in the CKD kidney.** CKD mice showed an increase in the Wingless-related integration site (Wnt) ligands mRNA expressions with an increase in β-Catenin protein expression. Downregulation of all the mRNA and β-Catenin protein expression was observed in both T3 and BAI treatment, with the maximum reducing effect observed in the combined treatment. **(a)** and **(b)** represents mRNA expressions of Wnt ligands in different groups. **(c)** western blot analysis of β-Catenin and creatine kinase 1 (CK-1); **(d)** and **(e)** represents densitometric analysis of β-Catenin and CK-1 protein expression, respectively. Data are expressed as means ± SEM from 3 independent experiments. ∗∗, and ∗∗∗ indicate the significance level at $p \leq 0.01$, and $p \leq 0.001$, respectively.

**Fig. 7. Effect of T3, BAI, and combined (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 higher reduction in the combined treatment. Data are expressed as means ± SEM from 3 independent experiments. ∗, and ∗∗∗ indicate the significance level at $p \leq 0.05$, and $p \leq 0.001$, respectively.

**Fig. 8. Effect of T3, BAI, and combined (T3 + BAI) on proinflammatory markers in the CKD kidney.** The protein expression of proinflammatory markers like nuclear factor kappa B (NFκB) and interleukin 6 (IL-6) increased significantly in CKD kidneys. As a kidney protective role, T3 and BAI reduced both NFκB and IL-6 expression, with a maximum reduction observed in the combined treatment. **(a)** western blot analysis of NFκB and IL-6; **(b)** and **(c)** represents densitometric analysis of NFκB and IL-6 protein expression, respectively. Data are expressed as means ± SEM from 3 independent experiments. ∗∗, and ∗∗∗ indicate the significance level at $p \leq 0.01$, and $p \leq 0.001$, respectively.

**Fig. 9. Effect of T3, BAI, and combined (T3 + BAI) on mRNA expression of Phosphate and Calcium transporter.** T3 and BAI treatment significantly protect the kidney and alter the mRNA expression, with a more pronounced effect in their combined treatment. **(a)** and **(b)** mRNA expression of NPT2a in different groups and treatments. **(c)** and **(d)** represents the mRNA expression of TRPV5. Data are expressed as means ± SEM from 3 independent experiments. ∗, ∗∗, and ∗∗∗ indicate the significance level 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 showed protective effect against CKD-induced cardiovascular damage with more pronounced effect observed by T3 treatment. However, a maximum protection has been observed in their combined (T3 + BAI) treatment. **(a)** and **(b)**, Calculated atherogenic index (AI) and coronary risk index (CRI) increased significantly in the CKD placebo group. All the

risk for the heart was reduced upon T3 and BAI treatment, with the maximum protective effect observed in the combined dose. **(c)** Histopathological analysis of kidney sections with the hematoxylin and eosin (H & E) staining indicating myocardium damage in CKD. **(d)** represents the calculated fibrotic area in heart section using Image J. ∗, ∗∗, and ∗∗∗ indicate the significance level at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

**Fig. 11. Schematic representation of bi-directional treatment using Triiodothyronine and Baicalein to upregulate and stabilize Klotho expression.**

## Table legends:

**Table 1.** Mice nucleotide sequence of 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 anaemia

**Table 5.** Effect of T3, BAI, and combined (T3 + BAI) on serum parameters for minerals and bone disorders