Journal of Advanced Zoology
ISSN: 0253-7214
Volume 42 Issue 02 Year 2021 Page 01-10

Impact Of Pcb On Kidney Injury Molecules -1 & Regucalcin Mrna Expression In The Kidney And Beneficial Role Of Antioxidant Vitamin C And E

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Abstract

Background: PCBs are global environmental contaminants that cause disruption of the endocrine system by increasing free radical production in humans and animals which will cause major disorders. Besides, the human body has several counteract mechanisms for oxidative stress by producing antioxidants - in situ or external supplements like vit C and E. This highlights the potential role of the antioxidants in preventing and repairing damages caused by oxidative stress.
Aim: The study was aimed at assessing the impact of PCB on kidney injury molecule-1 (KIM 1) and regucalcin mRNA expression in kidney tissue and also to study the effect of antioxidant vitamin C and E against PCB.
Method: For this study, Male albino wistar rats weighing 180-200 grams were used. Animals were divided into three groups i.e, control group, PCB induced group, PCB induced VitaminC and E treated group consisting of 6 animals each. At the end of treatment the total RNA isolation was done followed by quantification of RNA, cDNA- reverse transcriptase, Quantitative analysis-real time PCR was done to analyze the mRNA expression levels of KIM-1 and regucalcin. The triplicate analysis results of the experiments performed on control and treated rats were expressed as mean ± SEM. Results were analyzed statistically by one-way analysis of variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range test using Graph Pad Prism version 5. The results with $p<0.05$ level were considered to be statistically significant.

Result: In PCB induced animals there was significant increase in mRNA levels of KIM-1 and decrease in level of regucalcin. While vitamin C and E treated animals showed alternation in these mRNA levels to near normal as compared to the control group.
Conclusion: Our present findings show that PCB exposure causes detrimental changes in the renal tissue which may develop diabetic nephropathy by modulating the expression of KIM-1 and regucalcin molecules. Supplementation of Vitamin C and E have a protective role against PCB-induced changes.

Keywords: PCB, diabetic nephropathy, Kidney, KIM-1, regucalcin, Novel method, Innovative technology

INTRODUCTION :

Polychlorinated biphenyls, commonly known as PCBs, are a group of synthetic organic chemicals that were widely manufactured and used in various industrial and commercial applications from the 1930s until their ban in the late 1970s due to mounting evidence of environmental persistence and toxicity. These compounds are characterized by their chemical stability, lipophilicity, and resistance to degradation, properties that made them valuable for applications such as dielectric fluids in transformers and capacitors, hydraulic fluids, plasticizers, adhesives, and carbonless copy paper. However, these same properties have resulted in their persistent presence in the environment decades after production ceased, and they continue to be detected in soil, water, air, and biological samples worldwide. PCBs are accumulated in our body through the food chain, particularly through consumption of contaminated fish, meat, and dairy products, and due to their lipophilic nature, they accumulate in adipose tissue and are only slowly metabolized and eliminated. Once in the body, these compounds cause a variety of adverse health effects including neurotoxicities such as cognitive deficits, learning impairments, memory problems, and motor dysfunction, which have been documented in both human epidemiological studies and animal experiments. In particular, neonates and developing children are pondered as a high risk group for the neurotoxicity of PCB exposure, as their developing nervous systems are particularly vulnerable to disruption by these compounds, and exposure during critical developmental windows can result in lifelong deficits.

Diabetes and hypertension are important contributors to morbidity and mortality worldwide, representing major public health challenges that affect hundreds of millions of people and account for substantial healthcare expenditures and loss of quality of life. These conditions are caused by some combination of genetic predisposition and environmental factors, which may include exposure to persistent organic pollutants such as polychlorinated biphenyls (Everett, Frithsen and Player, 2011). Epidemiological studies have demonstrated associations between elevated PCB levels and increased risk of type 2 diabetes, insulin resistance, and hypertension, suggesting that these environmental contaminants may contribute to the growing burden of metabolic disease. The mechanisms underlying these associations are not fully understood but may involve disruption of endocrine signaling, induction of oxidative stress and inflammation, and interference with glucose and lipid metabolism.

Diabetic nephropathy is a serious diabetes complication that may progress to end-stage renal disease, a condition requiring dialysis or kidney transplantation for survival, and is associated with a high risk for premature death from cardiovascular and other causes. Diabetic nephropathy develops in approximately 20-40% of patients with diabetes and represents the leading cause of end-stage renal disease worldwide. The pathogenesis of diabetic nephropathy involves complex interactions between metabolic and hemodynamic factors, leading to structural and functional changes in the kidney including glomerular basement membrane thickening, mesangial expansion, podocyte loss, and eventually glomerulosclerosis and tubulointerstitial fibrosis. Early detection of diabetic nephropathy is critical for implementing interventions that can slow disease progression, and there is ongoing interest in identifying novel biomarkers that can predict which patients are at highest risk for progression. Kidney injury molecule-1 (KIM-1) and regucalcin serve as clinically useful predictors of diabetic nephropathy progression in patients with type 1 diabetes, offering the potential for earlier identification of at-risk individuals and more targeted interventions (Campion, Sanchez-Ferras and Batchu, 2017).

KIM-1 is a type 1 transmembrane glycoprotein comprising an extracellular portion with immunoglobulin and mucin domains, and a cytoplasmic portion with phosphorylation sites that may participate in intracellular

signaling. Ichimura first reported that KIM-1, which was shed into urine after acute kidney damage, is a sensitive and specific marker of renal tubular injury, with levels correlating with the severity of damage (Ichimura et al., 1998). Subsequently, it was recorded that over-expression of KIM-1 is also a marker for the long-term prognosis of chronic kidney diseases, with elevated levels predicting more rapid decline in renal function and progression to end-stage disease. Beyond its role as a biomarker, KIM-1 has important biological functions in the kidney. KIM-1 is associated with control of viral infections through its role as a receptor for certain viruses, regulation of autoimmunity and immune tolerance through effects on T cell function, and modulation of atopic diseases. KIM-1 plays a role in the removal of apoptotic cells through phagocytosis, functioning as a phosphatidylserine receptor that recognizes and promotes clearance of dying cells, thereby limiting inflammation and tissue damage. Its interaction with p85, the regulatory subunit of phosphoinositide 3-kinase, enhances cell autophagy to degrade KIM-1-containing phagosomes, linking the molecule to cellular quality control mechanisms (Yang et al., 2015). In kidney diseases characterized by protein overload, KIM-1 can be used to increase the phagocytosis of albumin by renal tubular epithelial cells, which alleviates the tubular damage caused by proteinuria and represents an adaptive protective response (Zhao et al., 2016).

Regucalcin is a protein that in humans is encoded by the RGN gene located on the X chromosome (Shimokawa, Matsuda and Yamaguchi, 1995). The protein encoded by this gene is a highly conserved, calcium-binding protein that is preferably expressed in the liver, kidney, and other tissues including brain, heart, and lung (Misawa and Yamaguchi, 2000). Studies indicate that this protein may also play a role in aging, with down-regulation of regucalcin expression observed in various tissues during the aging process, potentially contributing to age-related cellular dysfunction. This gene is part of a gene cluster on chromosomes that includes other functionally related genes, suggesting coordinated regulation (Yamaguchi, 2019). Regucalcin may regulate the effects of calcium ions on liver cell functions, including modulation of enzyme activities, regulation of cell proliferation and apoptosis, and maintenance of intracellular calcium homeostasis (Murata and Yamaguchi, 1998). In the kidney, regucalcin has been implicated in protection against cellular stress and maintenance of normal tubular function, making it a relevant target for investigation in models of nephropathy.

Glucose homeostasis is a complex process involving communication between multiple tissues including the pancreas, liver, muscle, and adipose tissue, in order to maintain the level of glucose in a precise physiological range without involving dietary nutrient ingestion during fasting periods (Indumathi et al., 2013). This intricate regulatory system involves hormones such as insulin and glucagon, as well as neural inputs and local paracrine signaling. Disruption of glucose homeostasis leads to hyperglycemia and the development of diabetes, with consequent complications including nephropathy, neuropathy, retinopathy, and cardiovascular disease. Environmental factors, including exposure to persistent organic pollutants such as PCBs, may contribute to the disruption of glucose homeostasis and increase diabetes risk.

As an antioxidant, vitamin C provides protection against oxidative stress-induced cellular damage by directly scavenging reactive oxygen species including superoxide, hydrogen peroxide, and hydroxyl radicals, thereby preventing oxidative damage to proteins, lipids, and DNA (Wu et al., 2019; Chen et al., 2019; Li et al., 2020; Babu and Jayaraman, 2020). Vitamin E, a lipophilic antioxidant, helps in the neutralization of lipid peroxidation products by integrating into cell membranes and interrupting the chain reaction of lipid oxidation, thereby preserving membrane integrity and function. Several authors have hypothesized that oxidative stress and lipid peroxidation play an important role in the pathogenesis of many deadly disorders like diabetes and cancer, with accumulating evidence supporting the involvement of oxidative damage in disease initiation and progression (Malaikolundhan et al., 2020; Han et al., 2019; Gothai et al., 2018; Veeraraghavan, Hussain, et al., 2021). The antioxidant properties of vitamins C and E suggest they may have protective effects against PCB-induced toxicity, which is thought to be mediated in part through oxidative stress mechanisms. Our team has extensive knowledge and research experience that has translated into high-quality publications in the fields of toxicology, oxidative stress, and kidney disease (Sathya et al., 2020; Yang et al., 2020; Rajendran et al., 2020; Barma et al., 2021; Samuel, 2021; Samuel et al., 2021; Tang et al., 2021; Yin et al., 2021; Veeraraghavan, Periadurai, et al., 2021; Mickymaray et al., 2021; Teja and Ramesh, 2020; Kadanakuppe and Hiremath, 2016; Vijayakumar et al., 2010; Kavitha et al., 2014; Lekha et al., 2014; Sahu, Kannan and Vijayaraghavan, 2014; Neelakantan et al., 2015).

The study was aimed to find out the impact of PCB exposure on KIM-1 and regucalcin mRNA expression in the kidney, and to evaluate the potential kidney protective role of antioxidant vitamins C and E against PCB-induced nephrotoxicity, as there is not much literature available about this specific topic. By investigating these relationships, this study seeks to contribute to the understanding of how environmental pollutants contribute to kidney disease and to identify potential nutritional interventions that could mitigate these effects, ultimately informing public health strategies for populations at risk of PCB exposure.

MATERIALS AND METHOD

Chemicals

All chemicals and reagents used in this study were purchased from Sigma Chemical Company St. Louis, MO, USA; Invitrogen, USA; Eurofins Genomics India Pvt Ltd, Bangalore, India; New England Biolabs (NEB), USA; Promega, USA. PCB was procured from Sigma Chemical Company St. Louis, MO, USA; Total RNA isolation reagent (TRIR) was purchased from Invitrogen, USA. The reverse-transcriptase enzyme (MMuLv) was purchased from Genet Bio, South Korea purchased from Promega, USA. Interleukin-1β and β-actin primers were purchased from Eurofins Genomics India Pvt Ltd, Bangalore, India and.

Animals

The present experimental study was approved by the institutional animal ethics committee (IAEC no.: BRULAC/SDCH/SIMATS/IAEC/12.2019/048). Adult male Wistar albino rats, weighing 180–200g, were obtained and maintained in clean propylene cages at the Biomedical Research Unit and Laboratory Animal Centre (BRULAC), Saveetha Dental College and Hospitals, Saveetha University, India) in an air-conditioned animal house, fed with standard rat pelleted diet (Lipton India Ltd., Mumbai, India), and clean drinking water was made available ad libitum. Rats were divided into 3 groups, each consisting of 6 animals.

Experimental Design

Group 1: Control (Vehicle control, rats were intraperitoneally (i.p.) administered with the vehicle (corn oil) for 30 days.
Group 2:Rats received PCB (PCB was dissolved in corn oil at a dose of 2mg/kg body weight (b.wt) intraperitoneally daily at 10:00 a.m. for 30 days.
Group 3:PCB and vitamin E (dissolved in olive oil at a dose of 50 mg/kg body weight), and vitamin C treated (100 mg/kg body weight dissolved in distilled water daily at 10 AM through gastric intubation for 30 days).
At the end of treatment, animals were anesthetized with sodium thiopental (5 mg/kg, i.p), and 20 ml of normal saline was perfused through the left ventricle, to clear blood from the liver, and other organs. Kidney was dissected out and used for the assay of various parameters.

Gene expression analysis by Real Time PCR

Isolation of total RNA

Total RNA was isolated from control and experimental samples using TRIR (total RNA isolation reagent) kit. Briefly, 100 mg fresh tissue was homogenized with 1 ml TRIR and the homogenate was transferred immediately to a microfuge tube and kept at -80°C for 60 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed for 1 min and placed on ice at 4°C for 5 min. The homogenates were centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 sec and placed on ice at 4°C for 10 min. The samples were centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 5min at 7,500 x g (4°C). The supernatant was removed and RNA pellets were mixed with 50 µl of autoclaved Milli-Q water and dissolved by heating in a water bath for 10 min at 60°C.

Quantification of RNA

Diluted RNA samples were quantified spectrophotometrically by measuring the absorbance (A) at 260/280 nm. 40 µg of RNA in 1 ml gives one absorbance at 260 nm. Therefore, the concentration of RNA in the given sample can be determined by multiplying its A260 by 40 and dilution factor. The purity of RNA preparation can be calculated using the ratio between its absorbance at 260 and 280 nm. A ratio of absorbance at 260/280 nm> 1.8 is generally considered as good quality RNA (Fourney et al., 1988). The purity of RNA obtained was 1.8.

Reverse Transcriptase – Polymerase Chain Reaction (RT – PCR)

RT-PCR is an approach for converting and amplifying a single stranded RNA template to yield abundant double stranded DNA products. 1. First strand reaction: Complementary DNA (cDNA) is made from the mRNA template using Oligo dT, dNTPs & reverse transcriptase. 2. Second strand reaction: After the reverse transcriptase reaction is complete, standard PCR (called the “second strand reaction”) is initiated. Principle RT-PCR is a method used to amplify cDNA copies of RNA. It is the enzymatic conversion of mRNA into a

single cDNA template. A specific oligodeoxynucleotide primer hybridizes to the mRNA and is then extended by an RNAdependent DNA polymerase to create a cDNA copy. First strand DNA synthesis The RT kit was purchased from Eurogentec (Seraing, Belgium). Reagents 1. 10X RT buffer: One vial containing 1.4 ml of 10X RT buffer. 2. EuroScript reverse transcriptase: One tube containing 75 µl of Moloney Murine leukemia virus reverse transcriptase (3750 U at 50 U/µl).

Quantitative Real Time PCR analysis

Procedure

Real Time PCR was carried out on CFX 96 Real Time system (Bio-Rad). The reaction mix (10 µl) was prepared by adding 5 µl of 2X reaction buffer, 0.1 µl of sense and antisense primer, 1 µl of cDNA and 3.8 µl of sterile water. The thermal cycler protocol was as follows: Initial denaturation at 95˚C for 3 min, followed by 40 cycles of PCR, denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 20 sec. All reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50-95˚C for each sample to determine the presence of multiple amplicons, non-specific products and contaminants. The results were analysed using CFX 96 Real Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin. The following primers were used in the present study:

RAT KIM-1: FW- 5" - ACTCCTGCAGACTGGAATGG - 3", RW- 5" – CAAAGCTCAGAGAGCCCATC– 3"; **Rat Regucalcin-** FW- 5'-AGATGAACAAA TCCCAGAT-3' , RW- 5'-TCACCCTGCATAGGAATAT-3'; **Rat β-actin-** FW – 5'- TACAGCTTCACCACCACAGC - 3' , RW– 5'- TCTCCAGGGAGGAAGAGGAT - 3'

Statistical analysis

The triplicate analysis results of the experiments performed on control and treated rats were expressed as mean ± SEM. Results were analyzed statistically by one-way analysis of variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range test using Graph Pad Prism version 5. The results with $p<0.05$ level were considered to be statistically significant.

RESULT:

Effect of antioxidant vitamins on kim-1 on kidney tissue

In PCB induced male wistar rats, there was a significant increase of mRNA in KIM-1 on contractions when it was treated with vitamin C and E, there was a decrease in levels of KIM-1 which was the same as the control group (fig. 1).

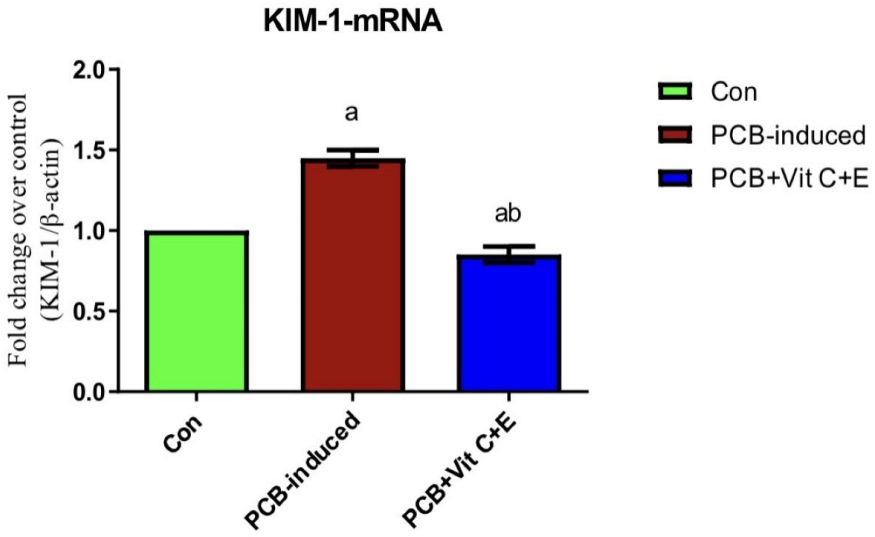

Figure 1: Bar graph represents the effect of PCB and vitamin C and E on the gene KIM-1. 'Green' depicts the control group, 'Red' depicts the PCB induced group and 'Blue' depicts the PCB treated with vitamin C and E.The expressions of KIM-1 were assessed by Real Time-PCR. Each bar represents Mean S.E.M of 3 observations representing 6 animals. Significance at $p<0.05$. a-compared with control; b-compared with PCB-induced.Green bar represents expression level of control rats, red bar represents expression level of PCB-

induced rats, and blue bar represents expression levels of PCB-induced rats which were later treated with Vitamin C and E.

Effect of antioxidant vitamins on regucalcin on kidney tissue

In PCB induced male wistar rats, there was a significant decrease of mRNA in regucalcin on contractions when it was treated with vitamin C and E, there was an increase in levels of regucalcin which was the same as the control group (fig:2).

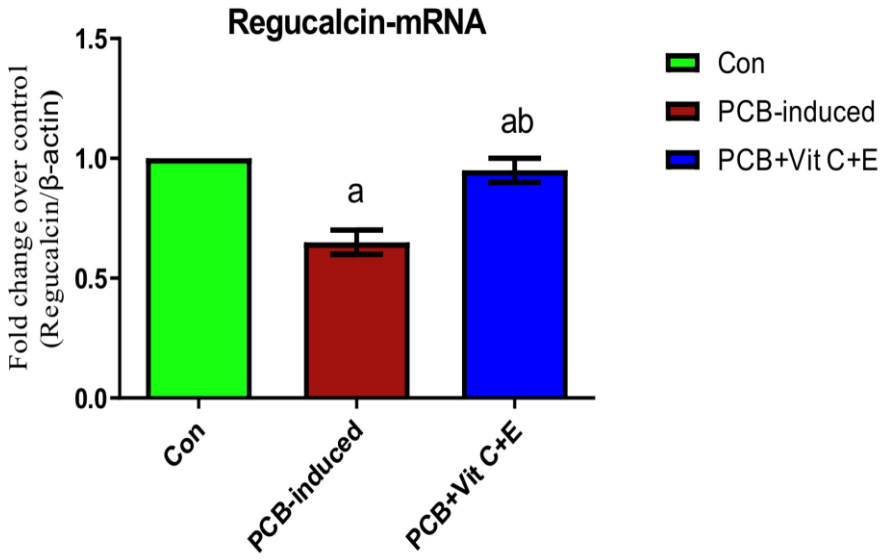

Figure 2: Bar graph represents the effect of PCB and vitamin C and E on the gene regucalcin. 'Green' depicts the control group, 'Red' depicts the PCB induced group and 'Blue' depicts the PCB treated with vitamin C and E. The expression of regucalcin was assessed by Real Time-PCR. Each bar represents Mean S.E.M of 3 observations representing 6 animals. Significance at $p<0.05$. a-compared with control; b-compared with PCB-induced.Green bar represents expression level of control rats, red bar represents expression level of PCB-induced rats, and blue bar represents expression levels of PCB-induced rats which were later treated with Vitamin C and E.

DISCUSSION:

Polychlorinated biphenyls (PCBs) are a category of chlorinated natural chemical compounds which are used for a spread of economic and industrial functions. Polychlorinated biphenyl is not one chemical, but a set of related chemical compounds which have not been located to occur certainly. Because the relative proportions of individual PCBs vary, counting on the intended use of the commercial product, analysis of PCB chemical styles found within the environment, offer clues that let the environmental forensics employee deduce the resources of infection (Johnson *et al.*, 1964).

PCBs are utilized in a lot of commercial and industrial applications like electrical, warmness switch, and hydraulic system; as plasticizers in paints, plastics, and rubber merchandise; in pigments, dyes, and carbonless reproduction paper; and much of other industrial programs. they're most usually cited with the help of their commercial trade names, like Aroclor and Kanechlor. However, PCBs surely include 209 character chemicals (Stackelberg and von Stackelberg, 2011). A number of the common applications of PCBs include: Scanning gadget: X-Ray screens, CT scanners, and ultrasonic scans all use electronic components with a view to feature. monitors: clinical tracking devices which include blood sugar display screen units, coronary heart fee and vital sign monitors all have electronic components inside. The cell has advanced a complex antioxidant system to urge obviate ROS, but imbalance between the manufacturing of ROS and antioxidant defenses leads to oxidative stress (Ford *et al.*, 2003). Protective role of lycopene against Aroclor 1254 has significant decrease of the GLUT 4 protein level in triceps muscles. C-2 deoxyglucose uptake showed much significant decrease in Aroclor 1254 treated rates alone(Williams *et al.*, 2013))

In a study done by (17) a distinctly found KIM-1 as a new marker for diabetic nephropathy. It is also named as HAVCR1 (Hepatitis an epidemic cell receptor 1) or TIM1 (T-cellular immunoglobulin mucin receptor 1), and is expressed inside the kidney, liver, and spleen. KIM-1 plays different roles via various molecular

objectives in immune illnesses and kidney injury. KIM-1 cares for HAV infections, autoimmunity, immune tolerance, and atopic illnesses. The urinary KIM-1 stage is carefully related to its tissue level, and correspondingly associated with kidney tissue damage. KIM-1 is not the most convenient early biomarker of acute kidney injury, but also features a capacity role in predicting the lengthy-time period renal outcome (Dachuri *et al.*, 2020). KIM-1 degrees regularly rose with the duration of diabetes. This manifestation of elevation of detectable level of serum KIM-1 without a doubt shows that with the event of diabetes and growing duration, there could also be disruption of renal function and boom in severity of renal irritation (nephropathy). Detection of KIM-1 in sufferers with a shorter period of diabetes (organization A-1) in their study extended tiers at six months to suit up shows the progression of kidney damage with time. Diabetes is said to be the deranged kidney functions that would stay undiagnosed usually. With the help of measuring the degrees at one-of-a-kind point in time and with distinct diploma of ailment severity in study members; they were ready to expose that KIM-1 ranges rises with the event of kidney disorder even when all other parameters including BUN or creatinine degree still be inside the everyday range (Panduru *et al.*, 2015). This finding is suggestive that because the kidney damage advanced, greater KIM-1 is secreted; therefore KIM-1 stages are often used for early detection of DN in diabetic sufferers. Whereas within the present study the PCB induced wistar rates (group-2) showed a big increase as compared with the control group, which is reversed on Vit C and E supplementation.

Regucalcin (RGN/SMP30) became initially observed in 1978 as a singular calcium-binding protein that does not comprise the EF-hand motif of calcium-binding area. Regucalcin has been shown to play a multifunctional role in cell regulation; retaining of intracellular calcium homeostasis and suppressing signs of transduction, translational protein synthesis, nuclear DNA and RNA synthesis, proliferation, and apoptosis in many mobile sorts. Moreover, regucalcin may additionally play a pathophysiological position in metabolic sickness. The expression of regucalcin is inspired via the movement of insulin in liver cells in vitro and in vivo and it is decreased within the liver of rats with type I diabetes induced by means of streptozotocin administration in vivo. Overexpression of endogenous regucalcin stimulates glucose utilization and lipid production in liver cells with glucose supplementation in vitro. Regucalcin is well-known which shows insulin resistance in liver cells. Deficiency of regucalcin induces an impairment of glucose tolerance and lipid accumulation within the liver of mice in vivo. Regucalcin could also be a key molecule in lipid metabolic sickness and diabetes (Yamaguchi, 2014). Within the present study, when PCB was induced into the male wistar rats we could evidently see a decrease within the level as compared with the control group, which is increased on Vit C and E supplementation. Antioxidants have a versatile role in improving our body metabolic functions as these are detoxification substances that help in removing the waste materials from our body through renal.

PCBs are considered as possible human carcinogens by the US environmental protection agency and WHO. There are various anti cancer drugs that are very effective towards the carcinogenic cells present in the body. Regucalcin gene expression has been demonstrated to be suppressed in various tumor tissues of animal and human subjects, suggesting a potential role of regucalcin in carcinogenesis(Yamaguchi *et al.*, 2018, 2021). Regucalcin plays a novel role as a suppressor in carcinogenesis of human patients with various types of cancer including pancreatic cancer, breast cancer, hepatoma, and lung cancer (Yamaguchi *et al.*, 2016).

Experimental research confirmed that antioxidant remedies ameliorated or inhibited diabetic nephropathy in rodents. There are reviews that glomerular hypertrophy is inhibited via intake of natural antioxidants extract which incorporates garlic, ginger and ginkgo in diabetic animals. Furthermore, glomerular hypertrophy is inhibited by means of antioxidants which incorporate Vit E and alpha tocopherol in diabetic rats (Kim, Gallaher and Saari Csallany, 2000). This research enhances the importance of antioxidants in our machinery life and biological magnification of PCB thus leads to an influence in genes such as KIM-1 and regucalcin which are biomarkers for diabetic nephropathy, the most common disorder leading to renal failure.

CONCLUSION:

Our present findings show that PCB exposure causes detrimental changes in the renal tissue which may develop diabetic nephropathy by modulating the expression of KIM-1 and regucalcin molecules. Supplementation of Vitamin C and E have protective roles against PCB-induced changes.

AUTHOR CONTRIBUTIONS

Author name: R Priyanka - contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

Author name: Gayatri R- contributed in study design, guiding the research work, manuscript correction.

Author name: J Selvaraj, V Vishnu priya, Kavitha S- study design, statistical analysis, manuscript proofreading and correction.

ACKNOWLEDGEMENT:

The authors express their gratitude to Saveetha Dental College & Hospitals for supporting and for successful completion of this project.

SOURCE OF FUNDING:

The present study was supported by the following agencies
- Saveetha Dental College,
- Saveetha Institute of Medical and Technical Science,
- Saveetha University
- Indusind bank PVT ltd, chennai.

CONFLICTS OF INTEREST:

The authors hereby declare that there is no conflict of interest in this study.

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