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Research Article

CHROMIUM AND ITK FORMULATION DIMINISH ARSENIC-INDUCED KIDNEY INJURY IN OBESE TYPE-2 DIABETIC RATS

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ABSTRACT: The present study was designed to evaluate the effect of pre-exposure to arsenic and chromium either alone or in combination for 90 days on renal dysfunctions associated with type-2 obese diabetes. Further, an attempt was made to assess the ameliorative potential of ITK (Indian Traditional Knowledge) formulation against these disorders. Induction of diabetes in obese rats significantly ($p<0.05$) increased the fasting blood glucose levels which were further increased in arsenic pre-exposed animals. Significant elevation in the kidney injury biomarkers (serum BUN, serum creatinine, urinary albumin, creatinine) were also observed in these groups of animals. In addition, the mRNA expression of cystatin-3 in kidney tissues was significantly ($p<0.05$) increased in the arsenic pre-exposed animals. On the other hand, pre-exposure to chromium produced nephro-protective effect as evidenced by the improvement in altered biomarkers of kidney injury. Further, chromium was also found to reduce or dampen the arsenic-induced kidney injury when administered concurrently in obese diabetic rats. Oral administration of one ITK formulation also supported the nephro-protective effect of chromium and may be recommended to reduce diabetes associated complication.

Key words: Type2 diabetes, Arsenic, Chromium, ITK formulation, Kidney injury, Rats.

INTRODUCTION

Diabetes mellitus (DM), commonly referred as diabetes, is a metabolic disorder characterised by high blood sugar levels over a prolonged period of time along with frequent urination and unexplained weight loss (Zimmet *et al.* 2001). The chronic state of hyperglycaemia leads to disturbance in the homeostatic systems of storage and mobilization of metabolic fuels including carbohydrate, fat and protein associated with absolute or relative deficiency in insulin secretion and/or its action (Nicolle *et al.* 2011). This global diabetes epidemic is chiefly due to type 2 diabetes as it makes up more than 90% of all diabetes cases (Tripathi and Srivastava 2006). Long-term complications from high blood sugar include heart disease, strokes, kidney failure, diabetic retinopathy leading to blindness (Zimmet *et al.* 2001). Diabetic nephropathy (DN) is reported to develop in 30-40% of patients with T2DM and has become a leading cause of end stage renal failure and death worldwide (Schena and Gesualdo 2005, Tanios and Ziyadeh 2012, Lizicarova *et al.* 2014).

Metals are inorganic elements occurring naturally and are present in small amount in living tissue and some of them (e.g. Mg, Mn, Cr, Fe and Cu etc.) are essential for maintaining some important biochemical pathways (Khan and Awan 2014). Chromium enhances the insulin receptor activity on target tissue, especially in muscle cells. Various studies have also reported that imbalance in these essential metals might adversely affect pancreatic islets and causes development of diabetes. On the other hand, toxic metals namely Pb, Ni, Cd and As were reported to be adequately present in the biological samples of type 2 diabetic patients and were associated with abnormal glucose uptake and altered glucose metabolism (Khan and Awan 2014). Human beings and animals are exposed to these toxic heavy metals from various man-made processes through contaminated water, air, soil and food and causes physiological, biochemical and histological disorders.

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Ground water contamination with arsenic is becoming an alarming threat for human beings and livestock throughout the world and is reported to be associated with several disorders like cancer and diabetes. Arsenic disrupts the glucose metabolism by modulating the signalling pathways of tumor necrosis factor-alpha (TNF-∝), mitogen activated protein kinase (MAP-kinase) and/or modulation of translocation of GLUT-4 to membrane which are mainly mediated by modulation of signal transduction factors including NF_KB, p38 mitogen-activated protein kinase (MAPK), tumor necrosis factor-∝ (TNF∝), phosphatidydylinositol-3-kinase (Somwar *et al.* 2002, Walton *et al.* 2004, Sriwijitkamol *et al.* 2006). On the other hand, trivalent form of chromium is reported to up-regulate glucose transporter (GLUT-4) translocation and thereby facilitating the glucose uptake. Thus, deficiency of chromium is reported to cause diabetes and associated complications (Khan and Awan 2014). However, their effect of diabetes-induced nephropathy is poorly understood.

Indigenous Traditional Knowledge (ITK) is the knowledge that people in a given community has developed over time and continues to develop it based on their experience over a long period of time, and which has been adapted to local culture and environment (Ghosh and Sahoo 2011). Globally, the concept of ITK formulations gained recognition through the World Conservation Strategy of International Union and Conservations of Natural Resources in 1980 followed by World Commission on Environment and Development, 1987 and United Nations Conference on Environment and Education in 1992 which recognized the existence of unique traditional knowledge in every culture, society and country. Since India has a long history and much enriched culture, there is abundant reservoir of indigenous knowledge in every part of the country (Pandey *et al.* 2017).

The ITK formulation used in the present study was composed of wheat, black cumin, barley and gum acacia. The phenolic compounds present in wheat (*Triticum aestivum* L.) have promising antioxidant, anti-inflammatory, anti-apoptosis properties (Ward *et al.* 2008,

Table 1. Different groups of animals used in present study.

Groups	Description	Treatment
Gr-I	Control (C)	Normal saline solution orally for 150 days
Gr-II	Obese Control (O)	Exposure to HFD and normal saline solution orally for 90 days
Gr-III	Obese + Diab. (O + D)	Exposure to HFD and normal saline solution orally for 90 days, and *i.p.* administration of STZ on 90th day
Gr-IV	Obese + Diab. + As (O + D+ As)	Exposure to arsenic (@ 38 ppb) in drinking water and feeding of HFD for 90 days, *i.p.* administration of STZ on 90th day
Gr-V	Obese + Diab. + As + ITK (O + D+ As + ITK)	Exposure to arsenic (@ 38 ppb) in drinking water and feeding of HFD for 90 days, *i.p.* administration of STZ on 90th day, administration of ITK orally (@ 3ml/kg b. wt.) daily from 90-150th day
Gr-VI	Obese + Diab. + Cr (O + D + Cr)	Exposure to chromium (@ 1ppm) in drinking water and feeding of HFD for 90 days, *i.p.* administration of STZ on 90th day
Gr-VII	Obese + Diab. + Cr + ITK (O + D+ Cr + ITK)	Exposure to chromium (@ 1 ppm) in drinking water and feeding of HFD for 90 days, *i.p.* administration of STZ on 90th day, administration of ITK orally (@ 3ml/kg b. wt.) daily from 90-150th day
Gr-VIII	Obese + Diab. + As + Cr (O + D+ As + Cr)	Concurrent exposure to arsenic (@ 3 ppb) and chromium (@ 1 ppm) in drinking water and feeding of HFD for 90 days, *i.p.* administration of STZ on 90th day
Gr-IX	Obese + Diab. + As + Cr + ITK (O +D + As + Cr + ITK)	Concurrent exposure to arsenic (@ 3 ppb) and chromium (@ 1 ppm) in drinking water and feeding of HFD for 90 days, *i.p.* administration of STZ on 90th day, administration of ITK orally (@ 3ml/kg b. wt.) daily from 90-150th day.

Vaher *et al*. 2010). Black cumin (*Nigella sativa*) has a wide range of pharmacological and biological activities including antihypertensive, antidiabetic, diuretics, anticancer, immunomodulator, analgesic, antioxidant, antimicrobial, anti-inflammatory, nephro-protective, gastro-protective, antioxytocic and anticonvulsant properties (Ahmad *et al.* 2013). β-glucan from cereals like barley has strong cholesterol and triglyceride lowering properties leading to reduced cardiovascular diseases (Lee *et al.* 2010). It modifies properties of chyme in the upper part of the gastrointestinal tract affecting gastric emptying, gut motility and nutrient absorption, which are reflected in lower postprandial glycemic and insulin responses because of its soluble fiber with viscous nature (Behall *et al.* 2006). Gum arabic is a soluble fermentable fiber and has shown hypoglycemic, antioxidant effects and also enhanced lipid metabolism in previous studies (Ali *et al.* 2009). Arabic gum by initiating the release of insulin from pancreatic beta cells, showed a significant hypoglycemic effect (Grover *et al.* 2002, El-Nagar 2017).

Though the effect of arsenic and chromium on renal disease has been reported, their potential role in modulating these diabetes-associated complications is largely unknown. Thus, the present study is aimed to evaluate the role of arsenic and chromium in diabetes-induced nephropathy as well as the efficacy of ITK formulations to ameliorate this diabetes-associated disorder.

MATERIALS AND METHODS

Experimental animals

Healthy adult male Wistar rats (160-170 g) were procured from Disease Free Small Animal House, LUVAS, Hisar, India. Rats were housed in polypropylene cages under 12-12 h dark-light cycle with free access to water and pelleted feed purchased from M/s Ashirwad Industries, Chandigarh, Punjab, India. They were acclimatized for a period of one week in the departmental laboratory animal house. The rats were randomly divided in nine groups having ten animals per group and subjected to different treatments as described in Table 1.

All the experimental protocols were undertaken following approval of the Institutional Animal Ethics Committee (IAEC) (Approval No.: IAEC/18/19 via letter No. 121/IAEC/18 Dated 25.09.2018).

Preparation of high fat diet

High fat diet (HFD) was prepared in our laboratory as described earlier (Suman *et al.* 2015) with minor modifications. Briefly, HFD was prepared by using normal pelleted feed (365 g), lard (310 g), mixture of vanaspati ghee and coconut oil in ratio of 2:1 (50 g), soyabean chunks and/or milk powder (250 g), yeast powder (1 g), sodium chloride (1 g), dl-methionine (3 g) and vitamin and mineral supplement (20 ml as syrup). All ingredients were mixed together and the mixture was then kept into a freezer to solidify before bringing them at room temperature. The mixture was then given to rats *ad-libitum* for a period of three months.

Preparation of hot aqueous extract of ITK formulation

For preparation of ITK formulation, 100 g each of ingredients *viz.* gum acacia, black cumin, wheat and barley were weighed and washed with fresh water. The ingredients together were soaked in water (300 ml). The mixture was heated up on a normal flame. The mixture was kept on flame for five minutes after start of boiling. After coming down at room temperature, the mixture was filtered using a sieve and the extract obtained (around 50 ml) was kept in air tight containers at 4 ^{0}C for further use. ITK formulation was given after 90 to 150 days, when diabetes was induced by Streptozotocin (STZ).

Type-2 diabetic model in rats

HFD-diet induced obese diabetic model was adopted as described earlier with some modification (Reed *et al*. 2000, Srinivasan *et al*. 2005). The HFD-fed obese rats were used for induction of diabetes at 90 days by injecting single dose of streptozotocin (@ 30 mg/kg b. Wt. i.p.). The dose of the streptozotocin was selected based on the

Table 2. Description of the primers.

Gene	Primer Sequence	Amplicon size (bp)	Annealing Temp. (°C)	Reference
Cyst-3	F5´-GCGTACCACAGCCGCGCCAT-3´ F-5´TGGGGCTGGTCATGGAAAGGACAGT-3´	149	63.2	Suzuki *et al.* (2014)
GAPDH	F5'-AAGGCTGAGAACGGGAAACT-3' R5'-TACTCAGCACCAGCATCACC-3'	101	62.0	Turchetti *et al.* (2015)

Table 3. Effect of pre-exposure to arsenic and chromium either alone or in combination on fasting blood glucose level in obese diabetic rats after administration of STZ (@30 mg/kg b.wt.).

Group	Description	Fasting blood glucose (mg/dl) After administration of STZ (@30 mg/kg b.wt.)					
		0 day	3 rd day	15th day	30th day	45th day	60th day
I	C	100.50^{d} ±0.56	106.60^{e} ±0.45	89.60^{g} ±0.63	99.70^{g} ±0.84	109.90^{g} ±0.80	101.00^{h} ±0.77
II	O	115.70^{a} ±0.47	105.10^{e} ±0.40	98.30^{f} ±0.61	101.50^{g} ±1.10	105.70^{g} ±0.57	111.00^{g} ±0.47
III	O +D	110.90^{b} ±0.54	311.70^{d} ±3.57	259.70^{e} ±3.28	299.10^{b} ±2.21	279.50^{b} ±2.49	253.50^{b} ±2.22
IV	O +D + As	112.10^{b} ±0.70	344.20^{b} ±1.20	270.20^{d} ±0.87	340.90^{a} ±1.12	326.10^{a} ±0.72	301.00^{a} ±1.14
V	O+D + As +I	104.80^{c} ±0.61	356.20^{a} ±0.94	326.40^{a} ±0.76	282.70^{d} ±0.95	262.50^{c} ±1.03	234.50^{c} ±0.92
VI	O +D +Cr	95.60^{e} ±0.52	321.50^{c} ±0.87	303.70^{b} ±0.93	290.20^{c} ±0.92	255.40^{d} ±1.05	228.80^{d} ±0.78
VII	O +D + Cr + I	90.80^{f} ±0.74	308.80^{d} ±0.78	290.20^{c} ±0.92	255.40^{f} ±1.05	228.80^{f} ±0.78	181.20^{f} ±0.72
VIII	O +D +As +Cr	102.40^{cd} ± 0.65	325.70^{c} ±0.90	305.30^{b} ±0.94	292.30^{c} ±0.88	275.10^{b} ±0.99	252.40^{b} ±1.21
IX	O +D +As +Cr +I	91.30^{f} ±0.73	324.60^{c} ±0.71	290.90^{c} ±0.79	265.00^{e} ±0.93	238.10^{e} ±0.78	193.40^{e} ±1.13

Data are presented as mean ± SEM, n=10. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test. Mean values with different superscripts within the same column are statistically significantly ($p<0.05$).

pilot experiment and earlier study performed in our laboratory (Pathak 2019). Streptozotocin (STZ) was dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5) and the buffer solution was injected intraperitoneally in rats to produce diabetes. Because pancreatic beta cells secrete insulin at high level following STZ injection, 10 % glucose solution was added to the drinking water of rats for next 24 h to avoid hypoglycemic shock. Seventy-two hours after injection of STZ, blood samples were collected for estimation of glucose using Accu-Chek Performa Glucometer (Roche diagnostics, USA). Rats with the glucose level of more than 200 mg/dl was considered as diabetic and further used for the study.

Estimation of biochemical parameters

Blood samples were collected periodically (on day 1, 3, 15, 30, 45 and 60 after induction of diabetes) as well as at the end of the experimental period (on 150 th day) from the rats of different experimental groups. Blood was collected through retro-orbital plexus from the inner canthus of the eye under anaesthesia (xylazine-ketamine) using capillary tubes or cardiac puncture under light ether anaesthesia. Blood samples from different treatment groups collected at different time interval following overnight fasting of animals were subjected to glucose estimation using Accu-Chek Performa, Roche glucometer and serum biochemical parameters, namely- blood urea nitrogen (BUN), uric acid and creatinine using commercially available kits (Span Diagnostic Ltd., India). The urine samples collected at the end of experiments were also used for estimation of creatinine and albumin level using commercially available kits. The biochemical parameters were estimated with the help of semi auto-analyser (Erba, USA) or UV-VIS spectrophotometer (ECIL, India).

mRNA expression study

At the end of experimental period, kidney samples from rats of different groups were collected in 0.1 % diethyl pyrocarbonate (DEPC) treated autoclaved PBS. After removing the adjacent fat and washing off the blood, the tissue samples were quickly snap-frozen in liquid nitrogen and stored in RNA later at -80ºC until further use. Total RNA was isolated using TRI reagent (Ambion, Thermo scientific) by following manufacturer's instruction. The samples were treated with RNase free DNase and DNase was subsequently inactivated by heating at 56ºC for 10 min and immediately chilled at 4ºC. The purity of RNA was checked by biophotometer (Eppendorf, USA). cDNA synthesis (from 1000 ng total RNA) was carried out from the mRNA present in the total RNA using Revertaid® First strand cDNA synthesis kit (Thermo Scientific, USA) using moloney murine leukemia viral reverse transcriptase enzyme by following the manufacturer's instructions.

Real-Time RT-PCR was performed using SYBR Green master mix (PowerUpTM SYBRTM Green master mix [2X]; ThermoFischer Scientific, USA). Each sample was run in duplicate in 10 µl reaction. The 10 µl reaction mixture consisted of 5 µl SYBR Green master mix, 0.25

Table 4. Effect of pre-exposure to arsenic and chromium either alone or in combination as well as the effect of ITK formulation on serum level of kidney function test parameters in type-2 obese diabetic rats.

Group	Description	BUN (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)
I	C	20.12^{f} ±1.20	1.99^{c} ±0.19	0.51^{f} ±0.05
II	O	29.06^{d} ±1.49	2.70^{c} ±0.21	0.52^{f} ±0.06
III	O +D	35.95^{bc} ±0.71	5.76^{b} ±0.12	3.41^{ab} ±0.15
IV	O +D + As	44.77^{a} ±2.56	8.27^{a} ±0.21	4.07^{a} ±0.24
V	O+D + As +I	30.22^{cd} ±1.97	5.74^{b} ±0.22	2.81^{bc} ±0.19
VI	O +D +Cr	27.31^{de} ±1.21	4.86^{b} ±0.60	2.07^{de} ±0.16
VII	O +D + Cr + I	21.22^{ef} ±1.07	2.74^{c} ±0.26	1.40^{e} ±0.13
VIII	O +D +As +Cr	38.17^{ab} ±1.40	5.18^{b} ±0.24	2.17^{cd} ±0.16
IX	O +D +As +Cr +I	26.03^{def} ±1.22	5.00^{b} ±0.36	1.80^{e} ±0.14

Data are presented as mean ± SEM, n=10. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test. Mean values with different superscripts within the same column are statistically significantly ($p<0.05$).

µl from 10 pmol/µl stock solution of each of the gene-specific forward and reverse primers, and 1.0 µl of cDNA and volume was adjusted to 10 µl with nuclease free water (NFW). The real-time PCR reaction was started with initial incubation at 95ºC for 2 min followed by 42 cycles of amplification with denaturation at 95ºC for 15 sec, annealing (temperature as mentioned in Table 2 for gene specific primer pairs) for 15 sec and extension at 72 ºC for 60 sec each. To assess the specificity of the amplified product, dissociation curve was generated at temperature of 60°C through 95°C. The results were expressed as threshold cycle values (CT).

Statistical analysis

Results were expressed as mean ± SEM with 'n' equal to number of animals used in the respective experimental protocols. The mean values from different groups were analyzed by one-way ANOVA followed by Tukey's post-hoc test using Graph-Pad Prism version 4.0 software.

To study the relative change in gene expression, the $2^{-\lhd\lhd CT}$ method was used as described previously by Livak and Schmittgen (2001). The formula used to calculate the fold change in gene expression was "fold change = $2^{-\lhd\lhd CT}$," [where $^{\lhd\lhd}C_T$ = (CT, target gene - C_T, GAPDH) treatment - (C_T, target gene - C_T, GAPDH) control]. The gene-specific amplification was corrected for the difference in input of RNA by taking housekeeping gene GAPDH to account. For treatment groups, evaluation of $2^{-\lhd\lhd CT}$ indicates the fold change in gene expression relative to healthy control *(i.e.*, fold change in healthy control = 1). The results were analyzed in comparison with the C_T (minimum threshold of amplification) value of the target gene and the reference gene (GAPDH).

RESULTS AND DISCUSSION

Diabetes mellitus is a metabolic syndrome characterized by defective control over plasma glucose either due to insulin deficiency or insulin resistance (Ferrannini 1998, Ye 2013). As per the epidemiological survey more than 350 million people around the world were affected by this widespread chronic disease in 2011 and its prevalence is expected to increase to approximately 550 million by 2030 (Whiting *et al*. 2011). Insulin resistance is central to development of type 2 diabetes mellitus and is commonly associated with heart disease, stroke, nephropathy, retinopathy and neuropathy. Current therapeutic approaches of diabetes have shown certain limitations, thus developing an alternative and potential therapeutic strategies become a thrust area of research for the scientific fraternity. Ethno-pharmacological approach provides low cost, long lasting therapeutic potential to treat diabetes mellitus and its associated complications. In the present study, we have investigated the effect of pre-exposure to arsenic (As) and chromium (Cr) on type-2 diabetes associated cardiovascular and renal dysfunctions and efficacy of ITK formulation against these complications.

Streptozotocin leads to production of antibodies which in turn causes apoptosis of pancreatic β-cells leading to impaired insulin synthesis and secretion (Simsek *et al*. 2012, Boslem *et al*. 2012). Fasting blood glucose levels (mg/dl) of rats from different groups on day 0, 3, 15, 30,

45, 60 after administration of streptozotocin (STZ @ 30 mg/kg b.wt, i.p.) are summarized in Table 3. Perusal of the data revealed that STZ administration significantly increased the fasting blood glucose level throughout the experimental period in the rats from Gr-III as compared to that observed in obese control (Gr-II) or healthy control (Gr-I) groups and these values were found to be more than 200 mg/dl. Pre-exposure to arsenic before induction of diabetes by STZ administration in Gr-IV rats further significantly ($p<0.05$) increased the fasting blood glucose level as compared to that observed in obese diabetic rats (Gr-III). However, pre-exposure to chromium either alone or in combination with arsenic significantly reduced the blood glucose level from 30th day onward, albeit, the level of the blood glucose in these animals were still found to be higher (>200 mg/dl). Treatment with ITK in Gr-V, VII and IX significantly reduced the fasting blood glucose level from 30 th day onward as compared to respective control (*i.e.* Gr-IV, VI and VIII, respectively). In consistent to the present observation, Rebecca *et al.* (2016) reported the efficacy of chromium in improving glycemic control by lowering blood sugar level. Further, chromium is reported to activate insulin receptor kinase and inhibit the insulin receptor phosphatase enzyme leading to increase in the phosphorylation of insulin and enhancement in the sensitivity (Davis *et al.* 1996).

Diabetic nephropathy is a severe complication of diabetes and the leading cause of death due to end-stage renal disease (ESRD). About 30% of diabetic patients suffer from kidney disease, proteinuria, and other severe symptoms, and eventually develop diabetic nephropathy and 53% of patients die from ESRD (Ge *et al.* 2019). The effect of different treatments on kidney function tests in the present study is summarized in Table 4. Perusal of the data revealed that, serum biochemical parameters related to kidney function test *viz.* blood urea nitrogen (BUN), uric acid and creatinine levels were significantly ($p<0.05$) increased in the obese diabetic rats (Gr-III) as compared to healthy control (Gr-I) or obese control (Gr-II) rats. Pre-exposure to arsenic in obese diabetic rats (Gr-IV) further significantly increased the BUN and uric acid level without further increasing the creatinine level as compared to obese diabetic rats (Gr-III), although these values were found to be still significantly higher in comparison to that observed in healthy control or obese control animals. Treatment with ITK formulation for 60 days significantly improved these kidney function test parameters (Table 4). Pre-exposure to chromium (Gr-VI) significantly ($p<0.05$) decreased the BUN and creatinine level in the serum of obese diabetic rats as compared to obese diabetic control (Gr-III) and ITK treatment further improved these biochemical parameters. Concurrent pre-exposure to arsenic and chromium did not produce further damage to kidney in obese diabetic rats as evident from the non-significant alteration in the serum level of BUN and uric acid with significant improvement in creatinine level as compared to obese diabetic rats (Gr-III). Treatment with ITK further improved the serum level of the parameters related to kidney function test in obese diabetic rats pre-exposed to arsenic and chromium in combination. Decrease in protein and increase in urea and creatinine levels in serum are the markers of kidney dysfunction (Latha and Daisy 2010). The elevated level of urea and creatinine in animal model of diabetes is suggested to be due to altered metabolic status following hyperglycemia and hypoinsulinemia characterized by reduced uptake of amino acids by the tissue, higher rate of proteolysis with reduction in protein synthesis leading to increase in urea production by the liver (Singh *et al.* 2018, Badawy *et al.* 2019). In the present study too, STZ produced nephropathy in obese rats as evidenced by the increase in the serum level of BUN, uric acid and creatinine along with corresponding increase in urine creatinine and albumin concentration. Impaired function of membrane barrier in the glomerulus in diabetic nephropathy often leads to increased excretion of albumin in urine, increased serum creatinine, and blood urea nitrogen (Tashiro *et al.* 2002).

Table 5. Effect of oral administration of ITK formulation for 60 days on urine level of albumin and creatinine in type-2 obese diabetic rats pre-exposed to arsenic and chromium either alone or in combination for 90 days.

Group	Description	Albumin (g/dl)	Creatinine (mg/dl)
I	C	1.60^{g} ±0.19	22.05^{e} ±0.96
II	O	2.45^{g} ±0.41	37.45^{d} ±1.02
III	O +D	15.75^{ab} ±0.61	49.27^{c} ±0.81
IV	O +D + As	18.69^{a} ±0.89	75.09^{a} ±1.52
V	O+D + As +I	13.76^{bc} ±0.78	57.56^{b} ±1.50
VI	O +D +Cr	9.47^{de} ±0.78	35.46^{d} ±1.05
VII	O +D + Cr + I	4.09^{fg} ±0.62	22.69^{e} ±0.91
VIII	O +D +As +Cr	12.00^{cd} ±0.77	51.29^{c} ±1.27
IX	O +D +As +Cr +I	7.01^{ef} ±0.73	38.87^{d} ±0.99

Data are presented as mean ± SEM, n=10. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test. Mean values with different superscripts within the same column are statistically significant ($p<0.05$).

Table 6. Effect of pre-exposure to arsenic and chromium either alone or in combination as well as the effect of ITK on mRNA expression of cyst-3 in kidney tissue.

Group	Description	Cystatin-3 (Fold Change)
I	C	$1.59^{a} \pm 1.24$
II	O	$55.92^{b} \pm 7.42$
III	O +D	$56.64^{b} \pm 3.21$
IV	O +D + As	$68.93^{b} \pm 15.50$
V	O+D + As +I	$29.89^{a} \pm 10.27$
VI	O +D +Cr	$3.38^{a} \pm 2.13$
VII	O +D + Cr + I	$6.28^{a} \pm 1.96$
VIII	O +D +As +Cr	$20.29^{a} \pm 6.27$
IX	O +D +As +Cr +I	$12.04^{a} \pm 8.01$

Data are presented as mean ± SEM, n=4. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test. Mean values with different superscripts within the same column are statistically significantly ($p<0.05$).

Further, to confirm the nephropathic effect, we evaluated the level of albumin and creatinine in the urine of the animals from different groups (Table 5). Perusal of the data revealed that, induction of diabetes in obese rats significantly ($p<0.05$) increased the urinary albumin and creatinine level and pre-exposure to arsenic in obese diabetic rats further increased the level as compared to obese control or healthy control. However, pre-exposure to either chromium alone or in combination with arsenic did not produce any significant alteration in the urinary albumin, creatinine level in the obese diabetic rats. Treatment with ITK formulation also did not produce any deleterious effect on urinary albumin, creatinine level.

Cystatin-3/Cyst-c (Cyst-3) is considered to be an early marker of diabetic nephropathy in patients with type-2 diabetes (You *et al*. 2013). It is a potent inhibitor of liposomal proteinases and probably one of the most important extracellular inhibitors of cysteine proteinase produced by nucleated cells. Cyst-c is freely filtered by the glomerulus and then absorbed by PCT, where it gets catabolised. A small amount of Cyst-3 is secreted in urine whereas its level is reported to be increased markedly during kidney dysfunction with reduced GFR (Lesley and Andrew 2019). To further assess the degree of kidney injury following pre-exposure to arsenic and chromium in obese diabetic rats, the mRNA expression profile of Cyst-3, one of the kidney injury biomarkers, were studied. As summarized in Table 6, the mRNA expression of renal Cyst-3 gene was significantly ($p<0.05$) increased in the obese diabetic rats (Gr-III; 56.64 ± 3.21 fold, n=4). Pre-exposure to arsenic for 90 days though did not further increase the mRNA expression of Cyst-3 in renal tissue, however, the value was found to be significantly higher as compared to control animals. Unlike arsenic, pre-exposure to chromium either alone (Gr-VI) or in combination (Gr-VIII) with arsenic did not increase the mRNA expression of Cyst-3 rather these values were found to be significantly lower in comparison to obese diabetic rats (Gr-III). Treatment with ITK further reduced the Cyst-3 level in the obese diabetic rats from the groups that were pre-exposed to arsenic and chromium either alone or in combination. Significant increase in the expression level of Cyst-3 in kidney tissue of rats from obese diabetic rats and/or arsenic pre-exposed obese diabetic rats indicated the presence of kidney dysfunction. Whereas chromium pre-exposure either alone or in combination with arsenic did not significantly increase the Cyst-3 level in comparison to their respective control implying its reno-protective effect. Administration of ITK formulated medicine also produced beneficial effect. The results of the mRNA expression studies of Cyst-3 are well corroborated with the findings related to other biomarkers of kidney injury as described above.

Chromium picolinate, an essential trace element, has long been used to treat type 1, type 2, gestational, and steroid-induced diabetes. This is popularly used as nutritional supplement for treating type 2 diabetic patients and diabetes-predisposed individuals (Jana *et al*. 2009). One of the major advantages of chromium-picolinate is its high stability and bioavailability in comparison to dietary chromium (Bailey *et al*. 2008). It is well known for its hypoglycemic activity as well as the ability to enhance insulin function and sensitivity (Broadhurst and Domenico 2006, Martin *et al.* 2008, Huang *et al*. 2014). However, its reno-protective role against diabetes-induced nephropathy is poorly understood. In the present study we have observed that pre-exposure to chromium significantly reduced the diabetes-induced kidney injury as evidenced from the findings of biochemical parameters. Recently, beneficial effect of chromium in diabetic nephropathy is also reported where the reno-protective effect of chromium picolinate was suggested to be attributed to its anti-oxidant potential and inhibition of TGF-β1/Smad2/3 expression (Qi *et al*. 2020). Possible existence of such mechanism in chromium-induced protective effect thus cannot be ruled out. Administration of ITK formulation also reduced the kidney damage produced by diabetes and/or arsenic-induced nephropathy in obese diabetic rats.

CONCLUSION

Based on the above findings, it may be concluded that pre-exposure to arsenic aggravated the kidney damage in obese diabetic rats while pre-exposure to chromium picolinate exhibited nephro-protective effect. Further, when chromium was administered concurrently with arsenic, it reduced the arsenic-induced damage to the kidney. This reno-protective effect of chromium was attributed to its ability to improve the specific biomarkers of renal injury, besides reducing the mRNA expression of cystatin-3. Administration of ITK formulation also enhanced the nephroprotective role of chromium.

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