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ORIGINAL ARTICLE

Diabetes and Obesity, Metabolic

Assessment of heavy metals by ICP-OES and their impact on insulin stimulating hormone and carbohydrate metabolizing enzymes

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Abstract

Arsenic (As) and cadmium (Cd) have recently emerged as major health concerns owing to their strong association with diabetes mellitus (DM). We aimed to investigate the heavy metals exposure towards incidence of DM at various enzymatic and hormonal levels. Additionally, association of As and Cd with Zinc (Zn, essential metal) was also evaluated. Spot urine samples were collected to assess As, Cd and Zn through ICP-OES. Serum was analyzed by assay method for fasting blood glucose, liver and renal function biomarkers. ELISA was performed to investigate the impact of heavy metals on HbA1c, α-amylase, DPP-IV, IGF-1, leptin, GSH, MDA, SOD, HDL, FFA, TG and interleukin (IL)-6. Association of heavy metals with DM was measured by odds ratio (OR) and level of significance was assessed by Chi-squared test. Unpaired student's *t*-test was used to compare DM-associated risk factors in heavy metals-exposed and unexposed participants. As and Cd were detectable in 75.4% and 83% participants with mean concentration of 75.5 ppb and 54.5 ppb, respectively. For As exposure, OR in the third quartile was maximum ie 1.34 (95% CI, 0.80 to 2.23), however the result was not statistically significant ($P > .05$). For Cd exposure, OR in the fourth quartile was considerably high, 1.62 (95% CI, 1.00 to 2.61), with a significant probability value ($P < .05$). Urinary Cd was negatively associated with Zn. As and Cd exposure increases the incidence of DM in the general population. Impaired hormonal and enzymatic levels in diabetic and non-diabetic exposed participants reflect the multiple organ damage by heavy metal exposure.

KEYWORDS

Arsenic exposure, cadmium exposure, inflammation, insulin resistance, interleukin-6

1 | INTRODUCTION

Essential metals (Zn, Cu, Co, Ni, Mg, Mn, Mo, and Ni) are required in minute quantities and are crucial for various biochemical and physiological reactions.[1] While, exposure of non-essential heavy metals (Cd, As, Hg, and Pb) is increasing day by day owing to rapid urbanization and industrialization. Cd and As are of great interest because the main threats to human health are associated with these metals as they are widely spread and pose a risk of multiple health disorders.[2-4]

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[Correction added on 17 June 2020, after first online publication: URL for peer review history has been corrected.]

Diabetes mellitus (DM) is considered as a multifactorial metabolic disorder accompanied with impairment of a number of biochemical reactions involved in carbohydrate and lipid metabolism. Essential transition metal (Zn) and non-essential heavy metals (As and Cd) are strongly linked with DM. Despite this, they also interact with each other and have key roles in the pathophysiology of DM.[5] Numerous free radicals (ROS) induced by hyperglycaemia are accountable for oxidative stress-induced β-cell destruction and insulin resistance.[6] Owing to the crucial role of oxidative balance in DM pathogenesis, studies on therapeutic intervention and prevention of DM focus on oxidative stress mechanisms by introducing various antioxidants. It is clearly indicated from previous research that heavy metals induce toxicity by elevating the oxidative stress level.[3,4,7,8] The involvement of Zn in the production of the antioxidant metallothionein (MT) has attracted much attention in the field of diabetic research to reduce metals-induced oxidative stress.[9-11]

Zinc contributes to insulin synthesis, crystallization, storage and secretion from β-cells. Moreover, it is also involved in translocation and action of insulin in the cells. Zn is also involved in insulin sensitivity by activating phosphoinositol-3-kinase/protein kinase B cascade. Owing to the insulinomimetic properties of Zn, it stimulates the uptake of glucose in various tissues. Moreover, Zn is also implicated in the suppression of pro-inflammatory cytokines and NF-kβ and protects the β-cells of pancreatic islets from death. All these properties of Zn may make it a potential candidate for DM prevention.[12,13] In addition to these, a number of studies have been done showing Zn to be effective in ameliorating DM-associated complications in various animal models.[14]

We aimed to evaluate the urinary exposure of heavy metals (As and Cd) in Pakistani population and investigated the association of these heavy metal with various risk factors of DM. We also intended to investigate the interaction of Zn with As and Cd exposure in Pakistani population. Furthermore, we also investigated the association of heavy metals with pathogenesis of DM by measuring the odd ratio and level of significance by Chi-squared test. Unpaired student's t-test was also used to compare DM-associated risk factors in heavy metals-exposed and unexposed participants.

2 | RESULTS

2.1 | Socio-demographic features of study participants

Among 724 participants, 451 were diabetic and 273 were non-diabetic. Arsenic was detectable in 546 (75.4%) participants while urinary As level was below the the detection range in 25.6% participants. Cadmium was detectable in 605 (83%) participants. Socio-demographic features of study participants with respect to As and Cd exposure are given in Figures 1 and 2 respectively. In the case of As exposure, strong association was observed in participants who were living in industrial areas, having low BMI and regular smokers (Figure 1). In the case of Cd exposure, a higher association was observed in the case of aged participants (>40 years) with higher BMI (≥30 kg/m^2) with OR (CI) of 2.36 (1.35 to 4.12) and 2.03 (0.77 to 4.30), respectively (Figure 2). After multiple regression analysis adjusted for education, gender, job and smoking status, urinary Cd level for model 2 (OR, 5.5714; 95% CI, 2.3800–13.0423; P = .000) and urinary As level for model 4 (OR, 19.6711; 95% CI,

Characteristics		Diabetic exposed/total	Non-diabetic exposed/total	OR (CI)	*p* value
Gender	Male	160/203	111/140	0.97(0.57 to 1.7)	0.92
	Female	183/248	92/133	1.30(0.79 to 2.0)	0.34
Age	18-39	112/150	70/88	0.76(0.40 to 1.4)	0.39
	40-59	206/261	125/170	1.30(0.86 to 2.1)	0.19
	≥60	25/40	8/15	1.45(0.43 to 4.84)	0.54
Residence	Rural	146/200	101/140	1.04(0.64 to 1.69)	0.86
	Urban	71/101	92/119	0.69(0.38 to 1.3)	0.24
	Industrial area	126/150	10/14	2.1(0.61 to 4.3)	0.23
Education	≤ high school	203/270	163/214	0.94(0.62 to 1.44)	0.80
	> high school	140/181	40/59	1.62(0.84 to 3.1)	0.14
Job	Employed	144/192	50/67	1.02(0.53 to 1.93)	1.00
	Unemployed	199/259	153/206	1.1(0.75 to 1.8)	0.52
Income	Low	102/127	63/84	1.4(0.70 to 2.6)	0.36
	Middle	171/221	82/110	1.16(0.68 to 1.98)	0.57
	High	70/103	58/79	0.76(0.40 to 1.46)	0.42
BMI	≤24.9	65/87	27/47	2.18(1.03 to 4.05)	0.04
	25-29.9	196/247	160/201	0.98(0.62 to 1.6)	0.95
	≥30	82/117	16/25	1.3(0.53 to 3.3)	0.55
Smoking	Yes	115/123	51/61	2.8(1.1 to 6.1)	0.03
	NO	228/328	152/212	0.9(0.61 to 1.31)	0.59

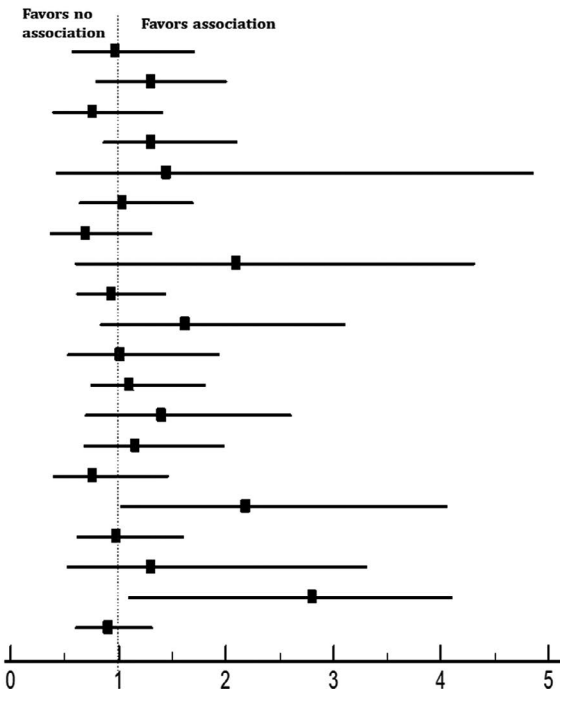

FIGURE 1 Socio-demographic characteristics of arsenic-exposed diabetic and non-diabetic study participants

Characteristics		Diabetic exposed/total	Non-diabetic exposed/total	OR (CI)	*p* value
Gender	Male	186/203	121/140	1.72(0.85 to 3.44)	0.12
	Female	197/248	101/133	1.22(0.74 to 2.02)	0.43
Age	18-39	116/150	77/88	0.48(0.23 to 1.02)	0.06
	40-59	236/261	136/170	2.36(1.35 to 4.12)	0.00
	≥60	31/40	9/15	2.29(0.64 to 4.19)	0.19
Residence	Rural	176/200	111/140	1.91(1.06 to 3.45)	0.03
	Urban	77/101	99/119	0.62(0.32 to 1.20)	0.16
	Industrial area	131/150	12/14	1.14(0.23 to 4.53)	0.86
Education	≤ high school	227/270	175/214	1.17(0.73 to 1.89)	0.50
	> high school	156/181	47/59	1.53(0.74 to 3.41)	0.23
Job	Employed	161/192	59/67	0.70(0.30 to 1.61)	0.41
	Unemployed	222/259	168/206	1.35(0.82 to 2.22)	0.23
Income	Low	110/127	71/84	1.18(0.54 to 2.58)	0.67
	Middle	193/221	88/110	1.72(0.93 to 3.18)	0.08
	High	80/103	63/79	0.88(0.43 to 1.81)	0.74
BMI	≤24.9	71/87	33/47	1.88(0.82 to 4.30)	0.13
	25-29.9	217/247	172/201	1.22(0.70 to 2.11)	0.48
	≥30	95/117	17/25	2.03(0.77 to 4.30)	0.14
Smoking	Yes	116/123	56/61	1.48(0.44 to 4.87)	0.52
	NO	267/328	166/212	1.21(0.78 to 1.86)	0.38

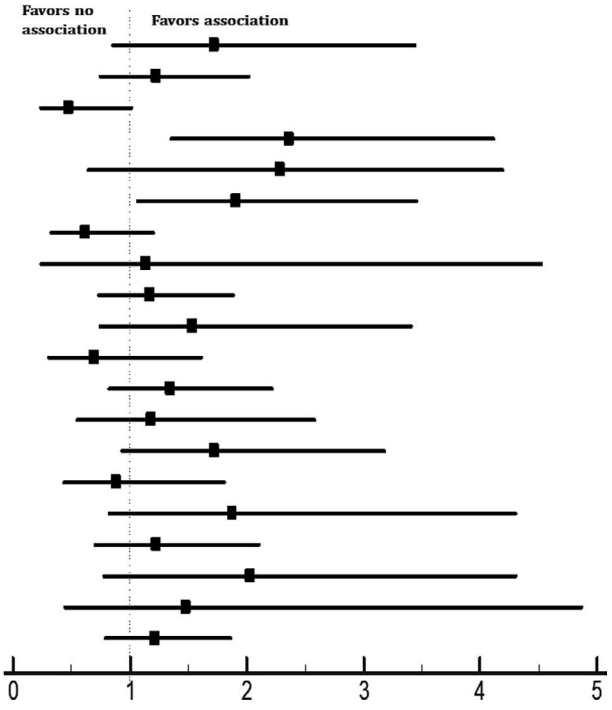

FIGURE 2 Socio-demographic characteristics of cadmium-exposed diabetic and non-diabetic study participants

2.6688–144.9920; *P* = .000) have shown association with DM (Table 1). As Pearson Chi-Square test shows a significant *P* value, so the two categories "As exposure" and "Cd exposure" have also been associated (Table 2).

2.2 | Exposure to heavy metals

The OR for DM in various quartiles comparing the exposed participants in the first quartile versus second, third and fourth quartiles has been given in Table 3. For As exposure, OR in third quartile was the maximum at 1.34 (0.80–2.23), however, the result was not statistically significant (*P* > .05). For Cd exposure, the OR of DM was negligible in the third quartile but it was slightly higher ie 1.23 (0.77–1.94) in the second quartile. The OR for the fourth quartile was considerably high, i.e. 1.62 (1.00–2.61) with a significant probability value (*P* < .05).

2.3 | Impact of heavy metals on DM-associated risk factors

After the measurement of urinary heavy metal concentration, various biomarkers reflecting the risk of DM, were compared with heavy metal exposure between the exposed and unexposed participants in both diabetic and control groups. The most important glycaemic biomarkers (RBG and HbA1c) were significantly high (*P* < .05) in As-exposed participants when compared with that of unexposed diabetic and non-diabetic participants. Additionally, As exposure has an association with serum α-amylase and IGF-1 level. Arsenic exposure also showed the decreased serum level of antioxidant defense biomarkers (GSH and MDA) and interleukin (IL)-6 (Table 4). Similar to the As exposure, Cd-exposed participants also showed significantly increased (*P* < .05) glycaemic control biomarkers. In addition to the elevated level of serum carbohydrate metabolizing enzymes (α-amylase and DPP-IV), Cd was also found to be positively associated with higher serum level of IL-6 (Table 5).

2.4 | Association of heavy metals exposure with Zn

We also investigated the beneficial impact of Zn on heavy metal toxicity in study participants. In As-exposed diabetic and non-diabetic participants, there was not a considerable association between urinary As and Zn level. In contrast to As, when we examined the association between urinary Zn and Cd level, a negative correlation was found between Cd exposure and urinary Zn level (Figure 3). The Spearman rank correlation coefficient (r) for Zn and Cd association was −0.347.

3 | DISCUSSION

Exposure to heavy metals, via food intake, drinking water and inhalation of dust particles, is of major public health concern, especially in underdeveloped countries. Sung and his colleagues conducted a meta-analysis on 38 research studies from various populations, in order to explore the association of As-exposure and risk of DM.

TABLE 1 Multiple logistic regression analysis for diabetes mellitus for urinary As and Cd level

Variables	Odds ratio (95% CI)	Chi-square	*P* value
Model 1			
As exposure	0.9667 (0.4955, 1.8857)	0.01	.921
Cd exposure	0.8721 (0.3911, 1.9447)	0.11	.738
Model 2			
As exposure	0.4708 (0.2377, 0.9325)	4.88	.027
Cd exposure	5.5714 (2.3800, 13.0423)	16.93	.000
Model 3			
As exposure	1.0214 (0.5266, 1.9813)	0.00	.950
Cd exposure	1.1335 (0.5156, 2.4923)	0.10	.755
Model 4			
As exposure	19.6711 (2.6688, 144.9920)	22.85	.000
Cd exposure	0.2234 (0.0265, 1.8868)	2.60	.107

Note: Model 1 includes the adjustment for education. Model 2 includes the adjustment for gender. Model 3 includes the adjustment for job and model 4 includes the adjustment for smoking status.

TABLE 2 CROSSTABS to test association between As exposure and Cd exposure

Parameters	Value	*df*	Asymp. Sig. y (2 sides)
Pearson Chi-square	254.306[a]	1	0.000
Continuity correction[b]	249.413	1	0.000
Likelihood ratio	240.119	1	0.000
Fisher's exact test			
N of valid cases	451		

They found that As enhanced the risk of DM in the general population with risk ratio of 1.71 (95% CI 1.32–2.23). Similarly, several studies conducted in various populations including United States,[15] Korea[16] and China,[17] suggest that a direct correlation exists between Cd exposure and risk of DM. Numerous studies have revealed that Cd-induced hyperglycaemia resulted in enhanced lipid peroxidation, reduced insulin secretion, enhanced activity of gluconeogenic enzymes and impaired insulin receptor signalling.[18,19]

Similar studies have also been conducted in Pakistan to assess the role of heavy metals (As and Cd) and essential trace metals in the pathogenesis of DM.[8,20-24] The key findings of our present study are also consistent with these studies. However, the level of heavy metals detected in our research was much higher as compared to the concentrations found in other studies; it might be that as Faisalabad is one of the largest industrial cities of Pakistan, there are more chances of heavy metals exposure. However, there are also few studies from other regions that did not support the association of As and Cd exposure with DM.[17,25-27] Such inconsistent findings might be attributable to diverse kinds of study subjects or some random methodological limitations including small sample size, suboptimal subject selection, and no adjustment of results to rule out other potential confounding risk factors.

In the kidney, the biological half-life of Cd is 6–38 years, while in the liver, Cd half-life is 4–19 years. The liver and kidneys together contain almost 50% of total the body Cd concentration.[28] In the current study, abnormal liver and renal function tests reflected the accumulation of Cd in these organs of exposed participants. Similarly, As has also been recognized for its hepatotoxic effects upon prolonged intake of contaminated drinking water.[29] Previous studies have shown that rapid industrialization in Pakistan is the major source of heavy metal contamination in drinking water.[30,31]

Cadmium disturbs the antioxidant defense mechanism by inhibiting SOD, CAT and GSH-Px with subsequently increased ROS level (Figure 4). Cadmium itself forms complexes with GSH and makes it inactive or reduces its scavenging activities.[32] Similarly, studies have also shown that As caused the decreased GSH and GSH-Px level most probably by making a complex with it.[33] This is because As and Cd have a strong affinity to bind with the sulfhydryl group present in GSH. Increased oxidative stress has been recognized as the major mechanism behind metal-induced metabolic disorders, particularly DM.[7] It is well documented that As and Cd increase the expression of pro-inflammatory cytokine (Figure 4). IL-6 is found to be involved in decreased insulin signalling and glucose-transporter (GLUT) translocation.[34-37] It is regarded as a major molecular pathway involved in heavy metal-induced insulin resistance.

Cadmium and Zn interaction is considered as one of the most recognized metal–metal interactions. Zn is abundantly found in pancreatic β-cells where it is involved in insulin synthesis and processing. Cd, competitive antagonist of Zn, utilizes the Zn transporters (ZnT, ZIP8 and DMT-1) for cellular influx and preferably accumulates in the pancreas after the liver and kidney. Serum α-amylase is used as bio-indictor to evaluate the pancreatic health. In the case of inflamed pancreas, there is a leakage of pancreatic enzymes, α-amylase and lipase, in to the blood. There was a slight but significant increase in α-amylase level in Cd-exposed participants. Our results are in accordance with the findings of Kalahasthi et al who recruited cadmium electroplating workers exposed to Cd in order to evaluate cadmium-induced pancreatic toxicity. An increased serum α-amylase level was found in these workers.[4]

Fernandez and his co-workers found a direct relationship between serum Zn level and DM. They conducted a systematic review of 16 clinical-based studies and concluded that Zn reduced the risk of DM by 41%. But conversely more interestingly, increased serum Zn level enhanced the risk of DM by 64%.[13] There is ample evidence which revealed that Zn supplementation to diabetic animal model enhanced the synthesis of metallothionein in liver, kidney, heart, and pancreas.[38] Cd is mainly excreted in the urine and the

TABLE 3 Odd ratios of diabetes by urinary As and Cd level

Parameters	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile
Urinary arsenic level (ppb)				
Arsenic conc.	<34.46	≥34.46–<68.52	≥68.52–<115.73	≥115.73
Diabetic exposed	76	89	92	86
Non-diabetic exposed	51	54	55	43
Odds ratio (CI)	1.0 (Reference)	1.10 (0.67 to 1.80)	1.12 (0.68 to 1.82)	1.34 (0.80 to 2.23)
P value	–	.68	.64	.25
Urinary cadmium level (ppb)				
Cadmium conc.	<50.46	≥50.46–<57.47	≥57.47–<63.88	≥63.88
Diabetic exposed	94	98	90	101
Non-diabetic exposed	65	55	59	43
Odds ratio (CI)	1.0 (Reference)	1.23 (0.77 to 1.94)	1.05 (0.66 to 1.66)	1.62 (1.00 to 2.61)
P value	-	.37	.81	.04

Note: Statistical analysis: Level of significance was evaluated by Chi-square test.

TABLE 4 Association of urinary As level with risk factors of DM among participants

	Diabetic			Non-diabetic		
Parameters	Exposed	Unexposed	*P*	Exposed	Unexposed	*P*
Glycaemic control biomarkers						
RBG (mg/dL)	327.5 ± 25.3	295.2 ± 17.7	<.05	135.5 ± 25.1	126 ± 28.6	<.05
HbA1c	11.52 ± 0.28	8.414 ± 0.13	<.001	6.1 ± 0.92	5.8 ± 0.78	<.05
Carbohydrate metabolizing enzymes						
α-amylase (ng/mL)	111.8 ± 9.76	70.90 ± 9.05	<.05	65.3 ± 14.05	58 ± 7.75	<.05
DPP-IV (ng/mL)	421.0 ± 38.99	395.8 ± 44.01	>.05	250 ± 30.21	271.8 ± 38.01	>.05
Insulin stimulating hormone						
IGF-1 (ng/mL)	180.3 ± 5.967	224.9 ± 27.19	<.05	305 ± 15.3	340 ± 11.8	<.05
Serum leptin level						
Leptin (ng/mL)	15.4 ± 3.8	13.8 ± 1.6	>.05	5.4 ± 3.2	4.8 ± 2.2	>.05
Oxidative stress biomarkers						
GSH (μmol/L)	0.7 ± 0.01	0.95 ± 0.02	<.05	1.1 ± 0.05	1.4 ± 0.1	<.05
MDA (mmol/L)	7.9 ± 1.1	6.7 ± 0.9	<.05	8.4 ± 1.6	7.1 ± 1.2	<.05
SOD (ng/mL)	29.3 ± 3.4	45 ± 5.8	<.001	37.7 ± 3.4	49.5 ± 7.1	<.001
Lipid profile biomarker						
HDL (mg/dL)	30.6 ± 10.3	33.1 ± 8.7	<.05	47.6 ± 6.5	56.1 ± 3.9	<.05
FFA (mg/dL)	31.2 ± 4.01	27.1 ± 3.1	>.05	20.5 ± 3.12	18 ± 2.45	>.05
TG (mg/dL)	208.9 ± 12.8	195.1 ± 9.8	<.05	142.3 ± 11.2	135.8 ± 13.7	<.05
Inflammatory biomarkers						
IL-6 (pg/mL)	10.6 ± 1.8	8.2 ± 1.6	<.05	4.8 ± 1.0	1.8 ± 1.2	<.001

Note: Statistical analysis: Two-tailed unpaired student's *t*-test was used to compare columns. Level of significance: probability value ($P < .05$) was considered significant.

Abbreviations: DPP-IV, dipeptidyl peptidase-4; FFA, free fatty acid; GSH, glutathione; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein; IGF-1, insulin growth factor-1; IL-6, interleukin-6; MDA, malondialdehyde; RBG, random blood glucose; SOD, superoxide dismutase; TG, triglyceride.

rate of excretion of unbound/free Cd is compared to the bound form. When Cd is tightly bound with metallothionein, it is majorly reabsorbed in the renal tubules resulting in reduced urinary Cd level.[28] The indirect involvement of Zn in Cd renal excretion might be a possible justification for the inverse association of urinary Cd and Zn in study participants. During a case-control study, Anetor and his co-workers also found an inverse relation between Cd and Zn.[5]

TABLE 5 Association of urinary Cd level with risk factors of DM among participants

	Diabetic			Non-diabetic		
Parameters	**Exposed**	**Unexposed**	***P***	**Exposed**	**Unexposed**	***P***
Glycaemic control biomarkers						
RBG (mg/dL)	310.5 ± 20.1	280 ± 18.6	<.05	125.5 ± 18.1	118 ± 19.6	<.05
HbA1c	11.1 ± 0.92	9.1 ± 0.78	<.05	6.15 ± 0.92	5.87 ± 0.78	<.05
Carbohydrate metabolizing enzymes						
α-amylase (ng/mL)	100.3 ± 11.05	76 ± 7.65	<.05	66.3 ± 12.05	57 ± 7.65	<.05
DPP-IV (ng/mL)	388 ± 40.21	401.8 ± 38.01	>.05	281 ± 31.21	255 ± 39.01	<.05
Insulin stimulating hormone						
IGF-1 (ng/mL)	205 ± 15.3	240 ± 10.8	<.05	309 ± 11.8	345 ± 16.3	<.05
Serum leptin level						
Leptin (ng/mL)	16.8 ± 2.9	17.2 ± 3.1	>.05	5.9 ± 1.2	6.78 ± 1.7	>.05
Oxidative stress biomarkers						
GSH (μmol/L)	0.75 ± 0.06	0.87 ± 0.1	<.05	1.2 ± 0.05	1.45 ± 0.1	<.05
MDA (mmol/L)	8.5 ± 1.6	7.6 ± 1.1	<.05	8.34 ± 1.6	7.21 ± 1.2	<.05
SOD (ng/mL)	36.8 ± 3.3	48.5 ± 6.1	<.001	36.8 ± 3.4	52.5 ± 7.1	<.05
Lipid profile biomarker						
HDL (mg/dL)	31.6 ± 10.5	34.1 ± 8.9	>.05	48.6 ± 6.7	55.1 ± 4.8	<.05
FFA (mg/dL)	32.5 ± 4.02	26.5 ± 0.05	<.05	20.25 ± 3.22	17.55 ± 2.25	>.05
TG (mg/dL)	210.2 ± 10.2	190.8 ± 13.7	<.05	151.3 ± 13.2	146.8 ± 12.7	<.05
Inflammatory biomarkers						
IL-6 (pg/mL)	12.8 ± 3.0	8.7 ± 1.1	<.001	4.9 ± 1.2	1.78 ± 1.3	<.05

Note: Statistical analysis: Two-tailed unpaired student's *t*-test was used to compare columns. Level of significance: probability value ($P < .05$) was considered significant.

Abbreviations: DPP-IV, Dipeptidyl peptidase-4; FFA, free fatty acid; GSH, glutathione; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein; IGF-1, insulin growth factor-1; IL-6, interleukin-6; MDA, malondialdehyde; RBG, random blood glucose; SOD, superoxide dismutase; TG, triglyceride.

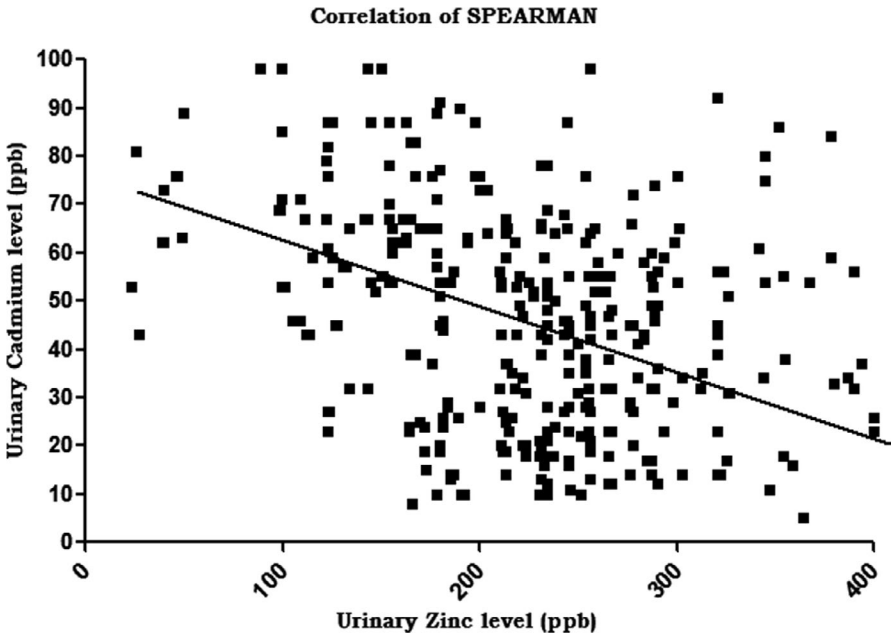

FIGURE 3 Association between urinary zinc and cadmium level among diabetic and non-diabetic study participants

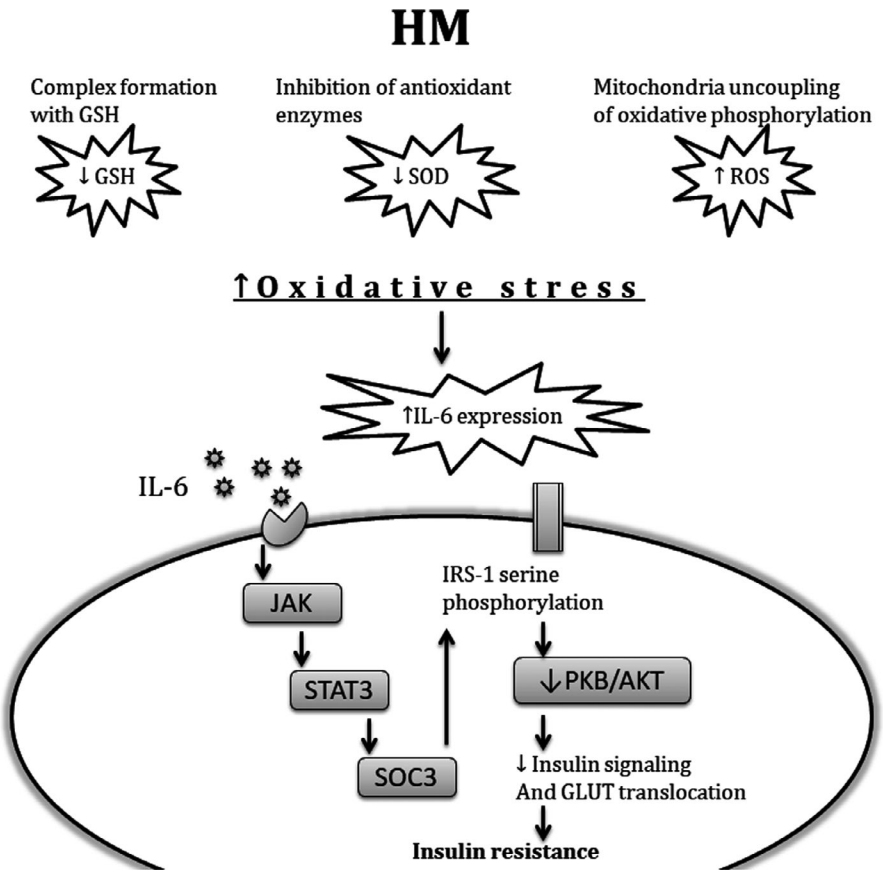

FIGURE 4 Schematic representation of heavy metal-induced insulin resistance via involvement of various molecular pathways

3.1 | Strength and weakness

We measured urine heavy metal concentration instead of blood. Blood heavy metal concentration reflects the recent exposure while urine level indicates the total body burden.[28] Total body burden is more important regarding their role in chronic diseases than recent exposure. A number of analytical tests have been performed regarding the risk factors of DM in exposed and unexposed participants in order to reveal the toxic effects of these metals on different body organs. However, further studies are needed with fairly large sample size to make the association more generalized.

4 | CONCLUSION

Our findings suggest that both the heavy metals (As and Cd) are associated with risk factors of DM. They may imbalance the redox system and disturb the level of enzymes crucial for carbohydrate metabolism. Elevated serum pro-inflammatory cytokines level along with impaired insulin stimulating hormone level reflects the increased susceptibility to develop metabolic disorders. Besides numerous beneficial effects of Zn supplementation in DM, it is important to ameliorate the toxic effects of heavy metals. While considering the preventive and therapeutic interventions for DM, it is also important to consider the heavy metals-induced toxicity resulting in various metabolic abnormalities.

5 | METHOD

5.1 | Study design and population

We randomly recruited 724 study participants regardless of age from both genders on the basis of their occupation, locality, and socio-economic status and collected urine and blood samples from the recruited participants between January 2019 and May 2019. We obtained ethical approval (IRB-GCUF-019298) from the Institutional Review Board (IRB) of Government College University Faisalabad (GCUF) to conduct this study. The study protocols were according to the ethical standards and procedures for research with human beings set by WHO.[39] The sample size was calculated by using Epitools-epidemiological calculator, with confidence level of 0.95 and $P < .05$.

5.1.1 | Inclusion and exclusion criteria

Participants were recruited depending upon doctor-diagnosed DM on the basis of positive preliminary screening tests and written consent was obtained from each study participant. Then, the questionnaire was filled to get data on socio-demographic characteristics of participants (like age, gender, job, residence, marital status, level of education and income status). Preliminary screening was done through questionnaire-based strategy. Initially 1150 participants were considered for this study, but after conducting

the interviews, 76 pregnant ladies, 45 participants reported the seafood intake within 24 hours and 15 cases of T1DM were excluded from this study. 290 participants were also excluded due to multiple diagnoses i.e. hepatic, renal and neurological disorders along with DM. Finally, 724 participants were qualified for this case-control study.

5.2 | Reagents and standard solutions for urine analysis

Reagents were obtained from Merck. All the chemicals were of supra pure quality. Highly pure deionized water was used for all experimental work. Solutions were kept in polyethylene tubes. Plastic tubes, autosampler cups, and all glassware were soaked in HNO_3 (10% v/v) for 24 hours, with subsequent five times rinsing by using Milli-Q water. Finally, they were dried and kept in laminar flow hood till experimental analysis. The working solutions of all analytes (As and Cd) were prepared on daily basis from certified standard solutions by using 2M HNO_3. These solutions were kept at 4°C.

5.3 | Conventional digestion of urine samples for ICP-OES analysis

The urine sample was collected in metal-free polypropylene containers by a clean catch method. While the blood sample was collected by 5 mL disposable syringe and transferred immediately into plain blood collection vial and kept in ice kit until further analysis. Urine was filtered prior to the experimentation. Duplicate 5 mL of each urine sample was put into 50 mL Pyrex flasks. A 10 mL volume of a recently prepared mixture of 65% HNO_3 and 30% of H_2O_2 (2:1, v/v) was put to every flask. Then, the sample containing the acid mixture was placed on an electrically heated hot plate at 80°C for 2–3 hours until a clear transparent digest was obtained. The final solution was stored in polyethylene tubes for determination of Cd and As through inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Teledyne Leeman Labs Prodigy 7). Blank digestion was also done. Samples for normal control were also treated as mentioned above. The samples (calibration blank, standards, reagent blank, and control sample) and matrix modifiers were introduced to ICP-OES. The calibration was verified periodically by analyzing the standard at the frequency of 10 readings. All experimental work was done at room temperature (25°C) by following well-established laboratory protocols.

5.4 | Estimation of glycaemic control biomarkers

Blood was centrifuged at 5000 rpm for 10 to 15 minutes at 4°C, and then, serum was separated. Serum samples were stored at −40°C till the experimentation. Random blood glucose (RBG) of study participants was measured at the time of sample collection by glucose strip method (INSTA ANSWER, Ref No. 907). The serum level of glucose and HbA1c (CAT No. SG-10984, Immuno Diagnostic) was estimated through the ELISA method by using a microplate ELISA reader (Bio Tek Instruments, Inc.) according to the instructions given in protocols at a wavelength of 450 nm.

5.5 | Estimation of liver and renal function biomarkers

Liver function and renal function biomarkers were estimated to assess the toxic effects of heavy metals on these organs where they are preferably deposited. AST (Itron, refer no 1002), ALT (Itron, refer no 1001), ALP (Itron, refer no 1003) and creatinine (Itron, refer no 1012) were determined by assay method by using microlab-300 chemistry analyzer.

5.6 | Estimation of carbohydrate metabolizing enzymes

The serum level of enzymes, found to be involved in carbohydrate metabolism notably α-amylase (CAT No. PRS-01604hu, PARS Biochem) and DPP-IV (CAT No. PRS-00535hu, PARS Biochem) were estimated by respective ELISA kit method by using microplate reader at 450 nm wavelength.

5.7 | Estimation of insulin stimulating hormone

Serum insulin-like growth factor-1 (IGF-1) level (CAT No. PRS-00825hu, PARS Biochem) was quantified to estimate the impact of EDCs on this hormone through ELISA kit method by using microplate reader at a wavelength of 450 nm.

5.8 | Estimation of serum leptin level

Serum leptin level (CAT No. PRS-00916, PARS Biochem) was quantified to assess the impact of EDCs on this hormone by the ELISA kit method by using the microplate reader at a wavelength of 450 nm.

5.9 | Estimation of oxidative stress biomarkers

The serum level of MDA (CAT No. SG-00097; Immuno Diagnostic), glutathione (GSH) (CAT No. SG-10391; Immuno Diagnostic) and SOD (CAT No. PRS-01469hu, PARS Biochem) were quantified through ELISA kit method by using microplate reader at 450 nm wavelength.

5.10 | Estimation of lipid profile biomarkers

The serum TGs (CAT No. SG-10999; Immuno Diagnostic), HDL (CAT No. SG-11051; Immuno Diagnostic) and FFAs (CAT No. SG-11107; Immuno Diagnostic) levels were estimated by using ELISA kit methods. The instructions and protocols for the estimations of these parameters were followed through the ELISA kit method by using microplate reader at wavelength of 450 nm.

5.11 | Estimation of the inflammatory biomarker

Interleukin-6 is considered an important inflammatory biomarker. Serum IL-6 level (CAT No. SG-10267; Immuno Diagnostic) was quantified through the ELISA kit method by using microplate reader at wavelength of 450 nm.

5.12 | Statistical analysis

All experiments were performed in triplicate and data are expressed as mean ± SEM. Statistical analysis was performed with the the help of GraphPad Prism 5 software (GraphPad Software Inc). We constructed univariate logistic regression models to identify independent factors associated with DM. Univariate analysis adjusted for education, gender, job, smoking and urinary heavy metal concentration. Crude and adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated. Unpaired student's *t*-test was used to investigate the significant differences of serum biomarkers between exposed and unexposed groups and probability value ($P > .05$) was considered significant. The Spearman rank correlation coefficient (*r*) was calculated to assess the association between heavy metals (As and Cd) and essential metal, Zn. MedCalc (MedCalc Software bvba, version 19.1) was used for calculation of odd ratios and drawing the forest plot. However, the Chi-square test was also applied to calculate the *P* value.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest for this article.

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