ORIGINAL RESEARCH PAPER
Volume - 11 | Issue - 08 | August - 2022 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

ALL-TRANS RETINOIC ACID ALLEVIATES ARSENIC-INDUCED ENDOCRINE DISRUPTION IN SWISS ALBINO MICE

Zoology

Joydeep Das — Research Scholar, Cancer Research Laboratory, Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Kolkata – 700019, India.

Zarqua Jamal — Research Scholar, Cancer Research Laboratory, Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Kolkata – 700 019, India.

Prof. Urmi Chatterji* — Cancer Research Laboratory, Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Kolkata – 700 019, India *Corresponding Author

ABSTRACT

Exposure to endocrine-disrupting chemicals (EDCs), such as arsenic, leads to severe health problems and surreptitiously accentuates stressful conditions in humans. Additionally, arsenic-induced endocrine stress leads to severe disturbances in glucose metabolism, mostly by disrupting the structure-function adroitness of the adrenal, thyroid and pancreas. Mechanistically, elevated levels of reactive oxygen species (ROS) generated by arsenic accentuate perturbation of cellular redox balance and eventually cell death. All-trans retinoic acid (ATRA), an active metabolite of vitamin A, is known for its anti-oxidant properties. Therefore, ATRA was used as a protection against arsenic-induced deteriorations of physiological conditions in mice. The present study reveals arsenic induced ROS generation in the adrenal and thyroid glands, accompanied by a decline in the activities of ROS scavenging enzymes, leading to disruption of the architecture of the tissues and induction of apoptosis therein, culminating in an imbalance in the hormonal secretions from the respective tissues. Structural damage to the pancreas accompanied with severe imbalances in glucose metabolism and associated biochemical parameters like glucose, pancreatic amylase and liver glycogen were also noted. Treatment with ATRA could efficiently reverse the deleterious effects induced by arsenic. Hence, ATRA can be used as an efficient nutraceutical which can lead to attenuation of endocrine stress induced by arsenic.

KEYWORDS

Endocrine disruption, arsenic, adrenal, thyroid, reactive oxygen species, apoptosis

INTRODUCTION

Endocrine disruptors are known to adversely affect human health, mostly by altering physiological control mechanisms. Endocrine-disrupting chemicals (EDCs) are compounds that disrupt the normal actions of all endocrine hormones including estrogens, androgens, thyroid, hypothalamic and pituitary hormones (Linehan et al. 2012; Elobeid and Allison 2008). Endocrine disruption by various environmental pollutants has attained scientific and public attention during the past few decades and has been a matter of grave concern (Buha et al. 2018). Arsenic, a heavy metal found ubiquitously across the globe, is one of the most hazardous EDCs which cause a wide range of health hazards including skin lesions, cancer, cardiovascular and immunological disorders (Choudhury et al. 2016; Sabir et al. 2019; Jamal et al. 2019).

Arsenic also acts on numerous endocrine organs and can alter the hormones involved in glucose regulation (Kulshrestha et al. 2014). Sodium arsenite has been shown to modulate the estrogen signaling pathway (Chatterjee and Chatterji 2010) and decreases circulating estradiol and gonadotropin levels in a dose- and time-dependent manner, mostly by generating reactive oxygen species (Chatterjee and Chatterji 2011, 2017). Arsenic, as a potent endocrine disruptor, can alter steroid hormone receptor-mediated gene regulation at very low, environmentally relevant concentrations in cell cultures and in animal models as well (Davey et al. 2008).

Reports suggest that arsenic exposure may also have a disruptive effect on the regulatory interactions between the hypothalamic-pituitary-adrenal axis (HPA) (Kinlein, Wilson, and Karatsoreos 2015) and the hypothalamic-pituitary-thyroid axis (HPT) (Sun et al. 2016, 2017), the two major axes related to stress and metabolic physiology. The hormonal and bioenergetic circuit is primarily involved with maintenance of cortisol, thyroid and insulin levels, and the organs that maintain those hormones, viz., the adrenals, the thyroid, and the pancreas (Lam 2020). The adrenal glands are chiefly responsible for counteracting stressful situations by secreting corticosteroids (McGaugh and Roozendaal 2000).

Arsenic is known to alter adrenocorticotrophic hormone (ACTH) levels in plasma (Delgado et al. 2000) and subsequently alter glucocorticoid activity (Thang et al. 2017). In addition, corticotrophin-releasing hormone (CRH) plays an important role in altering the thyrotropin-releasing hormone (TRH) and thyroid-stimulating hormone (TSH) secretion (Lam 2020). TRH and TSH eventually modulate downstream endocrine pathways viz., carbohydrate metabolism, gluconeogenesis and glycogenolysis (Tsigos and Chrousos 2002). Reports indicate that elevated levels of T_4 and T_3 and disruption of the hypothalamo-pituitary-thyroid axis (HPT) are associated with heavy metal intoxication (Ahmad et al., 2016). However, the mechanism involving the malfunctioning of the HPA axis, as well as the respective endocrine organs, in response to arsenic has not yet been elucidated.

Often a cross-talk occurs between two endocrine systems in order to alleviate stress conditions. The thyroid gland also responds to stressful conditions in response to elevated CRH. A delicate balance must therefore be maintained between stress hormones and cortisol for the proper functioning of thyroid gland. Serum TSH levels are positively correlated with serum cortisol levels (Walter et al. 2012).

The thyroid hormones TSH, along with T_3 and T_4, and the pancreatic hormone insulin, have a crucial role in metabolism. An imbalance in any one among these exerts a significant effect on the bioenergetic circuit. It is well established that glucose acts as the fueling agent for healthy metabolism. With chronic stress, the body requires more glucose to keep it geared up and also requires an increase in the basal metabolic rate. Prevention in the increase of BMR is caused by imbalances in the thyroid-pancreas-liver interaction, allowing stress to weaken the body.

Overly high levels of cortisol may render the cells to be thyroid resistant. When this kind of thyroid resistance occurs, it might lead to insulin resistance as well, one of the most common endocrinopathies of stress (Lam 2020). However, the mechanisms involving derangement of the HPA and HPT axes upon arsenic intoxication need to be discerned in detail. Hence, the present study has investigated the adverse effects of arsenic on the adrenal and thyroid architecture and demonstrated an important relationship between the hormones of the adrenal, thyroid and pancreas in the arsenic-exposed mice, with special emphasis to alterations in the respective downstream metabolic parameters.

Various phytochemicals, as well as other organic compounds, have been considered for their therapeutic efficacy against arsenic-induced stress (Singh et al. 2014; Ling et al. 2017; Jana et al. 2020). *All*-trans retinoic acid (ATRA) is one such promising compound for attenuating arsenic-induced toxicity (Chatterjee and Chatterji 2011, 2017). Retinoids, through their related receptors, have intense consequences

on cell development, differentiation and apoptosis, and assures disease attenuation and remedy (Siddikuzzaman, Guruvayoorappan, and Berlin Grace 2011). Retinoic acid signaling pathways contributes to numerous biological phenomena including tumorigenesis, inflammation, tissue damage and repair, developmental physiology and related diseases (Wei and Dong 2020; Ghyselinck and Duester 2019; Pawlikowski, Wragge and Siegenthaler 2019; Wu et al. 2019).

Additionally, ATRA is known to hinder cell multiplication by initiating cyto-differentiation, as well as, apoptosis in a few cell types. Till date, very few studies have focused on the ill-effects caused by exposure to arsenic, which led to various endocrine disorders (Madrigal et al. 2018; Grau-Perez et al. 2018).

We have reported earlier that ATRA ameliorated severe damages in the rat uterus caused by arsenic (Chatterjee and Chatterji 2011, 2017) by virtue of its defensive capability against arsenic-actuated oxidative stress and apoptosis. Since *All*-trans retinoic acid (ATRA) is known for its potent anti-oxidative and anti-apoptotic properties (Ahlemeyer et al. 2001), this study delineates the role of ATRA in alleviating the damages incurred by arsenic to the adrenal, thyroid and pancreas, and glucose metabolism, in Swiss albino mice.

MATERIALS AND METHODS

Chemicals and kits

Natrium meta arsenite ($NaAsO_2$); Cedarwood oil and D.P.X. mountant for microscopy were purchased from Merck (Germany). Paraplast paraffin pellets were procured from Leica biosystems (Netherlands). Amylase Assay Kit (Colorimetric), *all*-trans retinoic acid (ATRA), cleaved PARP and cleaved Caspase-3 antibodies, Optiblot ECL detect kit, and DCFDA were obtained from Abcam (UK). FITC-Annexin V apoptosis detection kit was from BioLegend (USA). Bax, Bcl2 and secondary antibodies were purchased from Santa Cruz Biotechnologies (USA). Reduced glutathione, glycine, sodium chloride, sodium dodecyl sulphate and anthrone were procured from Sigma Aldrich (USA). Mouse CORT ELISA kit was from Wuhan Fine Biotech Co. Ltd (China). Mouse glucagon and insulin ELISA kits were from Ray Biotech (USA).T4 and TSH ELISA kits were obtained from Thermo Fisher Scientific (India). Nor-epinephrine and epinephrine ELISA kits were procured from Labor Diagostika Nord Gmbh & Co., KG (Germany). Glucose Assay Kit was purchased from KEE GAD Biogen.

Animals

Male Swiss albino mice, weighing 25-30 g, were maintained under standard laboratory conditions (temperature 25±2°C, humidity 50±15% and 12 hr light-12 hr dark period) throughout the experiment. All animal experiments were done according to the rules of laboratory animal care (NIH Publication No 85-23, revised in 1985) and consent of the Institutional Animal Ethical Committee, Government of India (Registration Number 885/ac/05/CPCSEA). Indian Laws of Animal Protection (ILAP) were abided throughout the experiment. The animals were fed with standard food pellets and water *ad libitum.* They were allowed to acclimatize to the laboratory environment for 7 days before initiation of the experiments.

Experimental design and dose selection

$NaAsO_2$was administered in drinking water at the optimum effective doses of 5 ppm, 15 ppm and 300 ppm for 7 days followed by arsenic and ATRA co-treatment for 7 days. ATRA was dissolved in cotton seed oil and injected intraperitoneally at an optimum dose of 0.5mg kg^{-1} body weight (Chatterjee and Chatterji 2017). The doses of $NaAsO_2$and ATRA selected were based on earlier studies (Jamal et al. 2019, Chatterjee and Chatterji 2017), which conformed to environmentally relevant concentrations and acute doses.

Mice were divided randomly into eight different groups, each containing five animals (n=5). The groups were as follows: Group I (Control group, received arsenic-free water); Group II (received 5 ppm arsenic); Group III (received 15 ppm arsenic); Group IV (received 300 ppm arsenic), Group V (ATRA), Group VI (5 ppm arsenic+ATRA), Group VII (15 ppm arsenic+ATRA) and Group VIII (300 ppm arsenic+ATRA).

Histology

After specified treatment, mice adrenal, thyroid and pancreas glands were dissected out, weighed and processed for histology. 5µm paraffin sections were stained by standard hematoxylin-eosin double staining procedure (Chatterjee and Chatterji 2011). Structural alterations in the tissues were examined under a light microscope (Dewinter Optical Inc., India). The processed sections were analyzed for morphometric parameters using the Dewinter Caliper Pro software (Version 4.1).

Scanning electron microscopy

Adrenal and thyroid were harvested from the mice after dissection, minced into small blocks (1mm × 1mm) and fixed in 2.5% glutaraldehyde with 0.2 M phosphate buffer (PBS) for 2 hrs at 4ºC. The tissues were then washed with 0.2 M phosphate buffer followed by dehydration with ascending grades of ethanol. Tissues were finally placed in chilled acetone and air dried overnight. After air-drying up to the critical point, the tissues were coated with a gold-palladium alloy in a sputter coater (Quorum Q150TES) and finally viewed by the scanning electron microscope (ZeissEVO-18-Special edition, Germany) (Jamal et al. 2019).

Analysis of serum hormonal level

Serum was isolated from freshly drawn blood after arsenic treatment. Circulating corticosterone, epinephrine, TSH, T4, insulin and glucagon levels were assayed using specific ELISA kits, as per instructions of the manufacturers. ELISA kits for the determination of different hormones were based on competitive enzyme immunoassay and used a combination of specific antibodies to the hormones and hormone-HRP conjugates.

Analysis of biochemical parameters

Liver glycogen content was estimated calorimetrically by the method described by Hassid and Abraham (1957). The tissue (approximately 1gm) was placed in a pre-weighed centrifuge tube containing 3 ml of 30% KOH and boiled for 20-30min. When the tissue disintegrated, glycogen was precipitated by addition of 5 ml 95% ethanol. The precipitate obtained was dissolved in 1 ml of distilled water, re-precipitated with 95% ethanol and centrifuged at 3000 rpm for 10 min.

The glycogen precipitate was then dissolved in distilled water and the solution was used to estimate the quantity of glycogen. Briefly, to 0.1 ml of aliquot, 5 ml of anthrone reagent was added and mixed by swirling the tube. The tubes were heated for 10 min in boiling water, followed by cooling to room temperature and the absorbance was recorded at 590 nm. The readings were then compared with that of standard glycogen.

Serum glucose level was measured by the glucose oxidase-peroxidase (GOD-POD) enzymatic method (Trinder 1969). Glucose was oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. In a subsequent peroxide catalyzed reaction, the oxygen liberated was accepted by the chromogen system to give a red colored quinoneimine compound. The absorbance was measured at 505 nm and was found to be directly proportional to glucose concentration.

Serum pancreatic amylase activity was assayed using the amylase assay kit (Abcam). Amylase assay kit detects activity of α-amylase through two-step reaction. In the protocol, α-amylase cleaved the substrate ethylidene–pNP-G7 to produce smaller fragments that were eventually modified by α-glucosidase which led to the release of a chromophore. Serum of control and treated mice were mixed with reaction buffer and the absorbance was measured immediately at OD_{405} in a kinetic mode, every 2-3 minutes, for 30 minutes.

Intracellular ROS generation

Intracellular reactive oxygen species (ROS) generation was estimated by a flow cytometer, using the cell-permeant 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA), which is a chemically reduced form of fluorescein that acts as an indicator for ROS in cells. Acetate group of this compound is cleaved by intracellular easterases in presence of ROS and the nonfluorescent DCFDA is oxidized to the highly fluorescent adduct, 2', 7'-dichlorofluorescein (DCF). Cell suspension, prepared from adrenal and thyroid glands and stained with DCFDA was run on a FACS scan flow cytometer (BD FACS VERSE, USA) at 530 nm, and the results obtained were analyzed by the BD FACSuite™ software (Jamal et al. 2019).

Estimation of ROS scavenging enzymes

The activities of catalase (CAT) in adrenal and thyroid tissues were estimated by measuring the decomposition of H_2O_2 to H_2O and O_2 by CAT present in cell lysates, at a wavelength of 240 nm and expressed as µM of H_2O_2 U/mg of protein (Claiborne 1985). Superoxide dismutase (SOD) activity was evaluated according to the method of Marklund

and Marklund (1974), based on the self-oxidation rate of pyrogallol at 420 nm and inhibition of the same by SOD. One unit of enzyme activity corresponded to 50 per cent inhibition, expressed by units/mg protein. Reduced glutathione (GSH) assay was based on the protocol of Jollow et al. (1974). The method was based on the reduction of 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm. Reduced glutathione was assessed from a standard curve and indicated by µM/mg of protein.

Analysis of apoptotic cells by flow cytometry

Double staining for annexin V-FITC and propidium iodide (PI) was performed from cells of adrenal and thyroid of control and arsenic-treated mice. Cells were resuspended in annexin-binding buffer and staining reagents and incubated for 15 mins at room temperature. Distribution of cells was evaluated on a flow cytometer (BD FACS VERSE, USA) and was analyzed by the BD FACSuite™ software (Jamal et al. 2019).

Western blot hybridization

For protein analysis, excised tissues were homogenized in RIPA buffer containing protease inhibitors, and cell lysates collected by centrifugation at 14000 *x g* for 15 min. Total protein extracted from adrenal and thyroid tissues were resolved by SDS–PAGE and immunoblotted with antibodies against proteins that were involved in the apoptotic pathway. After incubation with respective secondary antibodies, binding was detected by enhanced chemiluminescence. Bands were analyzed using a Gel Documentation System (Gel Doc XR, Bio-Rad, USA). Band intensities were quantified with Image J software (Vers. 1.46r) and normalized using β-tubulin, the internal loading control (Jamal et al. 2019).

Statistical Analysis

Each experiment was performed at least in triplicates and mean ± SD were reported using a statistical software package (Graphpad Prism, Version 6.0). Every arsenic-treated group was contrasted with the control and the contrasts between the group mean values were evaluated by Student's *t*-test as all data sets were normally distributed. Statistical significance levels were set at *p*-values less than 0.05.

RESULTS

ATRA restores arsenic-induced structural and hormonal disruption in the adrenal gland

The adrenal gland from control mice showed regular histological features with clearly demarcated outer cortex and inner medulla. The cortex comprised parenchymal cells and was subdivided into zona glomerulosa, zona fasciculata and zona reticularis (Figure 1a and e). In contrast, the adrenals of arsenic-treated mice demonstrated cellular disruption and significant dose-dependent reduction in the cortico-medullary ratio ($p<0.001$; Figure 1b-d). However, restoration of the cortico-medullary ratio was observed when the mice were injected with ATRA intraperitoneally in all three groups of arsenic-exposed mice (Figure 1f-h). A section of the entire outer cortical and the inner medullary regions was studied by SEM.

The cortical layer, comprising three morphologically discrete zones was observed in the control adrenals, along with a clearly demarcated medullary region and intact capsule (Figure 1i and m). However, upon exposure to arsenic, the entire structural integrity of the gland was disrupted (Figure 1j-l). The capsule was disintegrated and the cortex and medulla were indistinguishable. Tissues were seen to be necrotized at higher doses of exposure (Figure 1k and l). Treatment with ATRA for 7 days led to restoration of the structural anomalies, along with re-formation of distinct cortical and medullary zones (Figure 1n-p).

In order to determine whether structural disintegration led to functional anomalies, the effect of arsenic on serum corticosterone and epinephrine levels was analyzed by ELISA in Swiss albino mice. Mice were subsequently exposed to different doses of sodium arsenite. As depicted in Figure 1q, serum corticosterone levels increased significantly ($p<0.01$ and $p<0.001$) with increasing doses of arsenic compared to the control group.

Upon treatment with ATRA, corticosterone levels reduced significantly ($p<0.05$) compared to the arsenic-treated mice (Figure 1q). Similarly, arsenic exposure led to a significant ($p<0.05$, $p<0.01$and$p<0.001$) increase in the serum epinephrine levels compared to serum of the unexposed mice (Figure 1r). ATRA treatment significantly reversed the levels of epinephrine to normal values (Figure 1r).

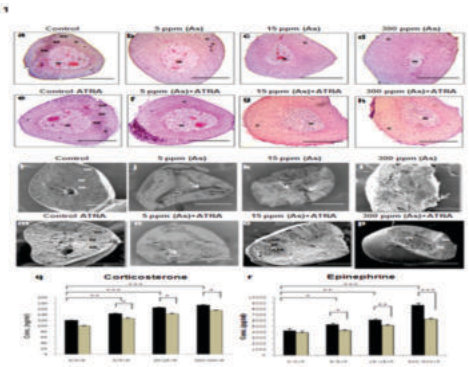

Figure 1. ATRA treatment reverses the arsenic-induced structural damage to the adrenal and causes the consequent restoration of the hormonal balance. (a-h)

Representative hematoxylin and eosin-stained sections of mice exposed to arsenic for 7 days followed by treatment with As+ATRA for 7 days. As treated histological sections **(b,c,d)** showed a decrease in the cortico-medullary ratio (10× magnification; scale bar 450µm) as compared to the control or 0.5 mg/kg ATRA-treated sections **(a and e)**. Significant recovery was noted in sections treated with ATRA following As exposure **(f, g and h)**. C, cortex; M, medulla; ZG, zona glomerulosa; ZF, zona fasciculate; ZR, zona reticularis. **(i-p)** Represent ultrastructural images of adrenal by scanning electron microscopy at a magnification of 200×. Micrographs depict morphological damage incurred to the adrenals of As-treated groups **(j-l)** as compared to the controls **(i and m).** As-induced structural anomalies were found to be restored upon treatment with ATRA **(n-p).** C, cortex; M, medulla; ZG, zona glomerulosa; ZF, zona fasciculata and ZR, zona reticularis; tissue degeneration, marked by arrows; scale bar 200µm. **(q)** Dose-dependent increase in levels of corticosterone was noted in As-exposed groups and upon ATRA treatment, a significant fall in the hormone level was seen. **(r)** ELISA results also revealed a dose-wise increment in the level of epinephrine upon arsenic exposure. Marked reduction in the circulating level of the same was noteworthy upon administration with ATRA. The data shown is a representative of experiments carried out in triplicates and represents mean ± SEM for all groups. *$p<0.05$; **$p<0.01$; ***$p<0.001$between control and As-treated groups and As-treated group versus As+ATRA-treated groups.

ATRA restores arsenic-induced structural and hormonal damages incurred to the thyroid

Tissue architecture of the thyroid gland from control mice revealed robust thyroid follicles, lined by distinct layer of epithelial cells, surrounded by dense fibrous connective tissue, which extended to support the follicles. Every follicle was filled with colloid substance (Figure 2a and e). Arsenic exposure led to dramatic alterations in the thyroid histology. Light microscopic observations showed significant hyperplasia of thyrocytes from 3- to 6- fold ($p<0.001$) compared to the control (Figure 2b-d), along with disruption of the epithelial lining. Follicles were mostly irregular with very scanty colloid materials or were vacuolated. Exfoliation of cells lining the lumen of follicles was also observed, together with congested blood vessels and vascularized stroma (Figure 2b-d). Infiltration of inflammatory cells in the follicles of thyroid was also observed in mice treated with 300 ppm arsenic where most of the follicles were devoid of colloid matter (Figure 2d). ATRA treatment not only restored the size and epithelial lining of the follicles, the compactness of the stroma was also apparent. Most of the follicles regained the colloid material as well (Figure 2f-h). Ultrastructural images of the thyroid of unexposed mice revealed a compact morphology with polyhedral outline of the thyrocytes (Figure 2i and m) lined by numerous microvilli on the cell surface. Upon exposure to increasing doses of arsenic, loss of cellular compactness, disintegration of the boundaries within the follicular cells, and eventually, degeneration of the gland were observed (Figure 2j-l). After treatment with ATRA, the compactness of the thyroids was seen to be restored, more specifically for the 5 and 15 ppm groups (Figure 2n-p).

The effect of arsenic on serum TSH and T4 levels of male Swiss albino mice was also resolved by ELISA. It was observed that exposure of

mice to 5 ppm, 15ppm and 300 ppm of sodium arsenite for 7 days significantly increased serum TSH levels by 1.8- (p<0.05), 2.6- (p<0.01) and 6.55-fold (p<0.001), respectively, compared to the control (Figure 2q). Upon treatment with ATRA, the TSH levels significantly reverted towards normal values (Figure 2q). T4 concentrations, on the other hand, were reduced to 4.08 µg/ml (p<0.05), 3.4 µg/ml (p<0.01) and 2.7 µg/ml (p<0.001) compared to the control value of 6.04 µg/ml, respectively (Figure 2r). ATRA treatment for 7 days significantly elevated the T4 levels by almost 1.5-fold (p<0.01) in all the three groups (Figure 2r).

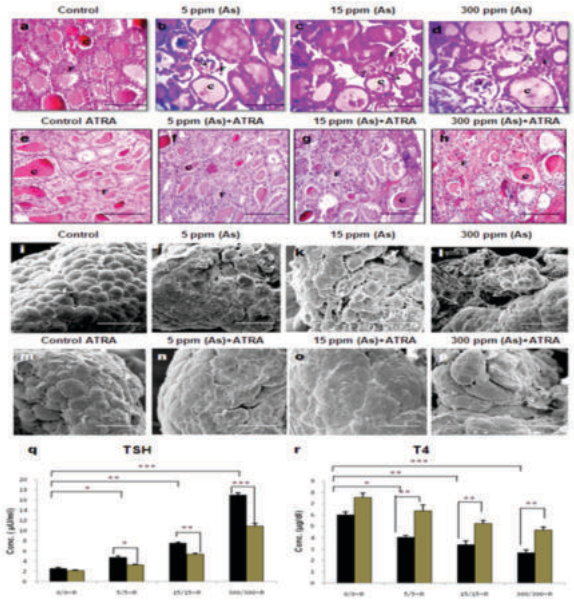

Figure 2. ATRA treatment restores arsenic-induced damage to the thyroid and scales back the hormonal imbalance. (a-h)

Histopathological changes in mouse thyroid was registered upon arsenic treatment **(b,c and d)** compared to the controls **(a and e)**. The damage incurred was found to be ameliorated upon ATRA injection following 7 days of arsenic exposure via drinking water **(f,g and h).** C, colloid; F, follicles; broken arrows indicate inflammatory cells in the follicles and solid arrows mark empty follicles,(magnification 40×, scale bar 100µm). **(i-p)** Represent the ultra structural images of thyroid by scanning electron microscopy at a magnification of 500×. **(j-l)** Depict the morphology of the thyroid of arsenic treated mice over controls **(i and m)**. **(n-p)** Show images of thyroid receiving ATRA treatment following arsenic.

Loss in the compactness of the tissue upon arsenic exposure was found to be restored after ATRA treatment**. (q)** Serum TSH levels of arsenic treated Swiss albino mice increased in a dose-dependent manner and was found to decrease significantly after ATRA-treatment following arsenic exposure. **(r)** Arsenic lowered the circulating levels of T4 by almost 1.5 and 2-folds. ATRA administration showed an ameliorating effect on arsenic-induced inhibition of circulating T4 levels. Data are representative of three independent experiments. Error bars represent S.D. from the mean of three independent experiments. *p<0.05; **p<0.01; ***p<0.001between control and As-treated groups and As-treated group versus As+ATRA-treated groups.

ATRA ameliorates arsenic-induced oxidative stress in the adrenal and thyroid

To confirm that the deteriorating effects were due to arsenic-induced oxidative stress, DCFDA staining of the cells was analyzed in a flow cytometer. The amount of ROS generated in the adrenal and thyroid of mice exposed to 5, 15 and 300 ppm sodium arsenite and *all*-trans retinoic acid was estimated. Concentration of ROS in the adrenal of arsenic-treated groups increased by 1.6-, 2.2- and 4.3-fold (p<0.05, p<0.01and p<0.001, respectively) over control mice (Figure 3a and b). A similar profile of ROS generation was observed in the thyroid, where ROS production increased by 1.3-, 1.4- and 4.5-fold (p<0.05 and p<0.001; Figure 3f and g) after exposure to arsenic. Treatment with ATRA showed a significant (p<0.05 and p<0.001) reduction in intracellular ROS levels in both the adrenal and thyroid glands (Figure 3a, b, f and g).

Fig.3

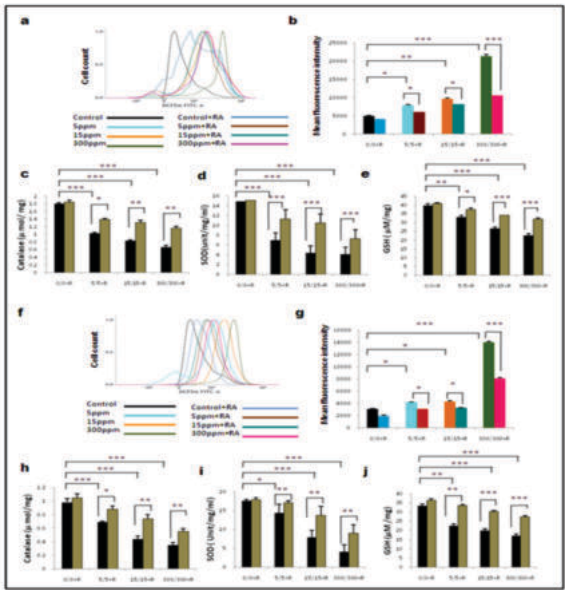

Figure 3. ATRA treatment ameliorates As-induced ROS generation and the disrupted enzymatic antioxidant system of adrenal and thyroid. (a, b and f, g)

Representative histogram profile of DCF fluorescence from adrenal **(a and b)** and thyroid **(f and g)** illustrating a dose-dependent increase in ROS generation upon exposure to acute arsenic for 7 days and a marked suppression of the same upon ATRA administration. **(c, d, e and h, i, j)** ROS scavenging enzyme assays from adrenal **(c, d, e)** and thyroid **(h, i, j)** showing a significant dose-dependent decrease in the activities of catalase (CAT), superoxide dismutase (SOD) and a depletion in the level of glutathione (GSH) compared to untreated controls. Significant recovery in the activities of CAT and SOD was noted accompanied with an improvement in the level of GSH upon ATRA treatment. Error bars represent S.D. from the mean of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 between control and As-treated groups and As-treated group versus As+ATRA-treated groups.

Concomitantly, dose-dependent reduction in the levels of ROS scavenging enzymes was also observed. CAT activity in the adrenals and thyroid reduced by almost 2.5-fold (p<0.001) after exposure to arsenic. Significant restoration of the enzyme activity was observed upon treatment with ATRA (up to1.5-fold; p<0.01) in both the glands (Figure 3c and h). Activity of SOD in adrenal and thyroid of mice receiving arsenic were seen to reduce up to 3- and 4-fold (p<0.05 and p<0.001), respectively. Treatment with ATRA successfully increased the activity of SOD (p<0.001 and p<0.01, respectively; Figure 3d and i). Oxidative stress was further intensified in the adrenal and thyroid of arsenic-exposed mice by deterioration in the levels of GSH up to 1.5-fold (p<0.001; Figure 3e and j). Animals which received ATRA injections up to 7 days showed significant recovery (p<0.05, p<0.01and p<0.001, respectively) compared to the mice which were exposed only to arsenic (Figure 3e and j).

ATRA protects the adrenal and thyroid glands from arsenic-induced apoptosis

The role of arsenic in stress-induced apoptosis was assessed and confirmed by flow cytometry using Annexin V-PI staining. Figure 4 demonstrates that arsenic insult led to apoptosis in the adrenal, as well as, the thyroid in a dose-dependent manner. Results indicated a dose-dependent increase in the early and late apoptotic cell population from 2.67% in the control to 19.09% in 5 ppm (p<0.001), 26.3% in 15 ppm (p<0.001) and 40.22% in 300 ppm (p<0.001) arsenic-treated mice adrenals (Figure 4a). In the thyroid, the percent increase of the same was from 9.3% of control mice to 10.92%, 12.7% (p<0.05) and 37.27% (p<0.001) in 5, 15 and 300 ppm arsenic-treated mice, respectively (Figure 4c). There was a perceptible reduction in the population of apoptotic cells, both in the adrenal (up to 1.33-fold; p<0.05) and thyroid (by3-fold; p<0.001) upon administration of ATRA (Figure 4a and c).

Fig.4

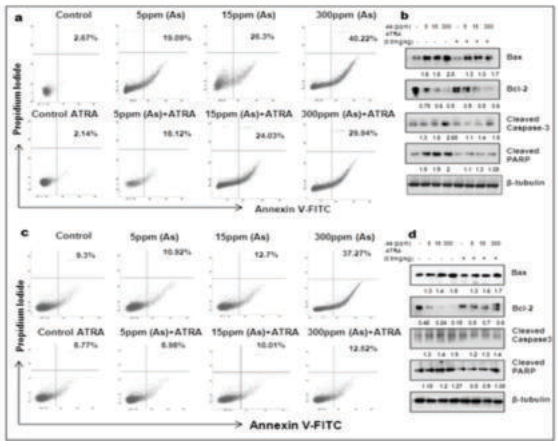

Figure 4. ATRA treatment inhibits As-induced apoptosis in mice adrenal and thyroid. (a and c)

Apoptotic profile of mice treated with sodium arsenite for 7 days followed by As+ATRA treatment for 7 days, adrenal **(a)** and thyroid **(c)**, quantified by Annexin V-FITC and PI double staining. **(b and d)** Immunoblots of apoptotic protein markers from adrenal **(b)** and thyroid **(d)** following 14 days of experimentaltenure. β-tubulin was used as loading control and respective fold changes are represented as ratio of net band pixel density of arsenic and As+ATRA treated groups to the control. Data are representative of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 between control and As-treated groups and As-treated group versus As+ATRA-treated groups.

In support of the above, significant increase in the expression of pro-apoptotic markers was also observed in the adrenal and the thyroid (p<0.05, p<0.01and p<0.001) of mice exposed to different doses of arsenic (Figure 4b and d). However, the animals which received ATRA injection up to 7 days showed significant reduction in the expression of the pro-apoptotic markers at the protein level compared to those of arsenic-treated mice (Figure 4b and d).

ATRA ameliorates arsenic-induced alterations in the mice pancreas

Histopathological examination of the pancreas of control mice revealed a well organized exocrine portion with compact lobular architecture, intact interlobular connective tissue and distinct interlobular ducts (Figure 5a and e). The endocrine pancreas indicated islets of Langerhans, interspersed between the pancreatic exocrine acini. The islets appeared lightly stained with well-demarcated boundaries in contrast to the surrounding acinar cells. Destruction and distortion of both the exocrine and endocrine portions of the pancreas were observed in arsenic-exposed mice, showing presence of necrotic and inflammatory cells. This was also accompanied by a loss in the compactness of the gland due to disintegration of the acinar cells (Figure 5b-d). Significant improvement in morphology of the islets of Langerhans and restoration of normal acinar cell population were observed in mice which received ATRA treatment (Figure 5f-h).

Fig.5

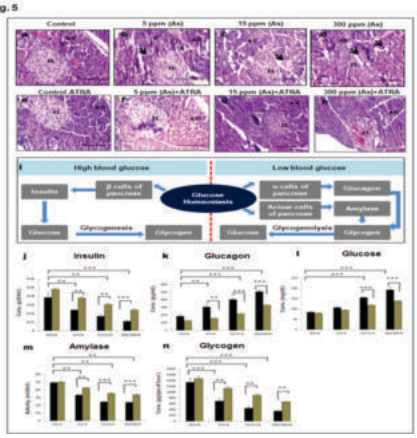

Figure 5. ATRA restores As-induced histological damages to the pancreas and normalizes glucose metabolism. (a-h)

ATRA reversed arsenic-induced alterations of glucose homeostasis

The schematic flowchart in Figure 5i illustrates the phenomenon of glucose homeostasis in brief. However, what happens to this circuit under conditions of heavy metal stress was to be determined. Our studies showed that exposure to arsenic led to significant alterations in the levels of serum insulin and glucagon. Compared to 0.038 µUL/ml in the control group (Figure 5j), serum insulin concentrations reduced to 0.023µUL/ml (p<0.01), 0.016 µUL/ml (p<0.001) and 0.011µUL/ml (p<0.001) in the 5 ppm, 15 ppm and 300 ppm arsenic-treated mice, respectively. ATRA administration increased the serum insulin levels significantly by 1.5 and 2-fold (p<0.01and p<0.001) compared to the mice which received only arsenic (Figure 5j). Concomitantly, sodium arsenite led to a significant increase in the serum glucagon levels after 7 days of treatment. Glucagon concentrations were increased by 1.6- (p<0.01), 2.16- (p<0.001) and 2.7- (p<0.001) fold in the 5 ppm, 15 ppm and 300 ppm arsenic-treated mice, respectively, in comparison to the control mice. After 7 days of ATRA treatment following arsenic exposure, dose-dependent decrease in the glucagon levels towards normal values was observed (Figure 5k).

Serum glucose levels of arsenic-treated mice increased significantly compared to the control mice, as shown in Figure 5l. The basal concentration of glucose in the control group was

Representative hematoxylin and eosin-stained cross-sections of pancreas from all experimental groups after treatment for 14 days. Magnification 40×; scale bar 100µm. **(a and e)** Control sections; **(b-d)** Arsenic treated sections depicting changes in acinar cells as well as a dose-dependent disruption of islets of Langerhans. **(f-h)** ATRA treated sections, indicating a significant recovery in tissue architecture and restoration of the islets to a great extent as compared to the As-treated ones. Solid arrows indicate degeneration of the tissue; AC, acinar cells; IL, islets of Langerhans. **(i)** Schematic representation of glucose metabolism **(j)** Serum insulin, **(m)** pancreatic amylase and **(n)** liver glycogen estimation revealed a significant dose-dependent decrease in the arsenic treated groups and a subsequent increase in the same upon exposure to ATRA. **(k)** Serum glucagon and **(l)** glucose was found to be elevated in all the three groups receiving different arsenic doses as compared to control. Amelioration in the levels of the same was evident upon administration of ATRA. Error bars represent S.D. from the mean of three independent experiments. *p<0.05; **p<0.01; ***p<0.001 between control and As-treated groups and As-treated group versus As+ATRA-treated groups.

84.97mg/dL. Serum glucose levels of mice exposed to arsenic increased to 106.96 mg/dL in the 5 ppm-treated group, which was slightly above the normal range of 80-100 mg/dL. However, glucose concentrations increased significantly to 156.31 mg/dL in the 15 ppm treated mice and 193.43mg/dL in the 300 ppm treated groups (p<0.001). ATRA administration following arsenic insult significantly (p<0.001) reduced the levels of glucose towards normal values in the 15 and 300 ppm groups (Figure 5l).

Pancreatic amylase activities were noted to reduce up to 2-fold over control (p<0.01) in arsenic-treated mice. A dose-dependent recovery in the activity of amylase by almost 1.5-fold (p<0.01and p<0.001)) was observed after ATRA treatment (Figure 5m).

Liver glycogen concentrations of the arsenic-treated mice were also altered radically compared to the control group. Glycogen levels were reduced by 1.9-, 2.9- and 3.7-fold (p<0.001) in the 5, 15 and 300ppm doses, respectively. Significant amelioration by 1.6-fold in 5 ppm group and almost 2-folds in 15 and 300 ppm groups (p<0.01) was observed after ATRA administration (Figure 5n).

DISCUSSION

Arsenic has been proved to be a potent endocrine disruptor (Chatterjee and Chatterji 2010, 2011), although its mechanism of action on different endocrine organs necessitated further investigation in order to implement therapeutic options. The fact that the responsiveness to this metalloid varies in different species and organs may be considered as an indication of multiple mechanisms of action (Ventura-Lima, Bogo, and Monserrat 2011). It is known to generate a variety of stress responses in mammals, including metabolic abnormalities, which eventually lead to apoptosis (Chatterjee and Chatterji 2017; Jamal et

al. 2019). Alterations in the ultrastructure of cells exposed to arsenic imply underlying disruption of functional elements responsible for consequent physiological disorders.

Stress responses, elicited by arsenic, target different endocrine glands which are involved in maintaining physiological homeostasis of an organism. Research related to understanding the effects of stress have been carried out mostly in murine models. Acute stress is generally seen in study durations for one week (Stroth and Eiden 2009). Increasing evidence indicates that disruption of the HPA axis can lead to deregulated stress response phenotypes which affect the physiology of the organism, and is known as allostatic load. High allostatic load can enhance vulnerability of the organism to toxic challenges induced by heavy metals like arsenic (Kinlein Wilson, and Karatsoreos 2015)

Interestingly, stress responses involve the HPA, as well as, the HTA axis. Therefore, acute stress responses can manifest in both the adrenal and the thyroid glands (Sun et al. 2016). The initial stress response of the adrenal gland is hypertrophy of the cells of the cortex (Harvey and Sutcliffe 2010), which occurs as a result of increased ACTH secretion, with increased levels of serum corticosterone as a direct response to toxicity. Adrenal medullary response to acute stress includes degranulation of chromaffin cells and increased secretion of epinephrine (Maronpot Boorman, and Gaul 1999). Although the involvement of the HPT axis with regard to stress has not been studied extensively, increasing evidence suggests that thyroid hormones also participate in response to chronic stress (Frick et al. 2009). Reduction of thyroid hormones has been observed in the serum of stressed animals, confirming that stress induces an alteration of the thyroid axis (Cremaschi et al. 2000). In addition, interaction between the HPA and HPT axes under stress has indicated that excess of glucocorticoid suppressed the central thyroid axis, whereas modulations of thyroid hormones regulate adrenocortical function through changes in the hepatic glucocorticoid metabolism (Wondisford 2015).

Significant damaging effects of arsenic on the adrenal and the thyroid, the two important components of the HPA and the HTA axis, were relevant. Striking alterations were seen in the respective tissue architecture. Additionally, increased corticosterone and epinephrine levels were documented which might have occurred owing to arsenic-induced structural anomalies of the adrenal gland. The effect of the metalloid was radical on the morphological and functional aspects of the thyroid gland as well, and exposure to arsenic was found to be associated with dose-dependent increase in TSH, and concomitant decrease in T_4 levels.

The most likely cause of damage incurred to the endocrine glands might be the generation of ROS, since it is reported to be the most important cause of arsenic toxicity. Skewing of the balance between ROS generating systems and anti-oxidant scavenging enzymes mostly culminates in an oxidative stressed condition (Marrocco, Altieri, and Peluso 2017). Our data confirmed elevated ROS generation in the adrenal and thyroid of arsenic-exposed mice, accompanied by potential damage to the ROS scavenging system. Furthermore, increased percentages of apoptotic cells, supported by over expression of Bax, cleaved caspase 3 and cleaved PARP after arsenic exposure, validated that oxidative damage finally culminated in cell death, thus aggravating the disruptive effects of sodium arsenite on the respective endocrine glands. ATRA treatment, however, appreciably counteracted the toxic effects of arsenic and restored the redox balance in the adrenal and thyroid of mice, preventing cell death therein.

The glucose level present in the blood is determined by the balance between the rate of glucose entering and leaving the circulation. Basal glucose homeostasis, chiefly regulated by insulin and glucagon, is maintained as a closed feedback loop involving the pancreatic islet cells, liver and peripheral tissues, including the muscle and adipose. When blood glucose concentrations are high, the liver and the skeletal muscles are signaled to remove glucose from the circulation and store it in the liver as glycogen, by a process named glycogenesis. When blood glucose concentrations are low, the liver is signaled to add glucose to the circulation by a process glycogenolysis, with the help of amylase, majorly pancreatic. Direct participation of the adrenal cortex in the control of carbohydrate metabolism has been confirmed, since adrenalectomy has been reported to lead to disturbances in carbohydrate metabolism (Long, Katzin, and Fry 1940). In addition, it is well established that thyroid hormones are important mediators of glucose homeostasis (Abdulmahdi, Alwachi, and Al-Lehibi 2013). Although the exact mechanisms involved are still unclear, a number of recent findings have concluded that several genes involved in glucose metabolism are regulated by thyroid hormones via binding to the thyroid hormone receptor (Boelen 2009). Subsequently, we investigated the effects of arsenic on the structure and function of the pancreas, since the islet hormones have a direct bearing on serum glucose levels, followed by its effects on pancreatic amylase and liver glycogen content. Predictably, in addition to disruption of the exocrine and endocrine pancreas, the level of insulin reduced significantly and that of glucagon increased, leading to a state of hyperglucagonemia. In healthy individuals, insulin suppresses alpha cell function and inhibits glucagon secretion. After exposure to arsenic, absence of the restraining influence of insulin on glucagon production led to hyperglucagonemia, along with increase in blood glucose levels. Increase in blood glucose and relative hyperglucagonemia in turn perturbed liver glycogen metabolism and eventually depleted liver glycogen levels in our experimental animals. In addition, as a result of arsenic-induced disintegration of the exocrine pancreas, the pancreatic amylase levels were also reduced in a dose-dependent manner, a condition which is indicative of pancreatitis. Serum amylases have been widely used for the diagnosis of acute inflammatory state of the pancreas (Oh et al. 2017). As the secretory capacity of the pancreas reduced during the course of pancreatitis, serum amylase values also reduced according to the residual functional capacity (Oh et al. 2017), as confirmed by our findings. Our studies further confirmed that treatment with ATRA helped in recovering the hormone levels towards normal values and induced the development of allostasis in Swiss albino mice.

Summary

In summary, this study illustrated that ATRA effectively improved the pathological disruption of endocrine glands, namely thyroid, adrenal and pancreas, which occurred as a result of exposure to arsenic. Results indicate a new pharmacological use for ATRA that may be attributed at least in part to the restoration in the hormonal balance through reduction of oxidative stress, subsequently resulting in the reduction of apoptosis in the mice adrenal and thyroid. ATRA also led to amelioration of the exocrine and endocrine pancreas, leading to a restoration of glucose metabolism. Taken together, since ATRA effectively improved the physiological health of the animals, it could be applied for therapeutic purpose, especially for people living in arsenic-infested regions.

Acknowledgement

We would like to acknowledge the central instrument facility of Centre for Research in Nanoscience and Nanotechnology, University of Calcutta, for flow cytometry and SEM studies. We are also thankful to DST-FIST, UGC-SAP, UGC-UPE and DST-PURSE for infrastructure support.

REFERENCES

[1] Abdulmahdi, R., Alwachi S.N., Al-Lehibi, K. I. (2013), "Detection for Pre- diabetes in Hypothyroid Patients." International Journal of Advance Research, IJAR, 1(8), 423-430.
[2] Ahlemeyer, B., Bauerbach, E., Plath, M., Steuber, M., Heers, C., Tegtmeier, F., and Krieglstein, J. (2001), " Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level." Free Radical Biology and Medicine, ELSEVIER, 30(10), 1067-1077.
[3] Ahmad, F, Liu, X., Zhou, Y., Yao, H., Zhao, F., Ling, Z., Xu, C. (2016) , "Assessment of thyroid endocrine system impairment and oxidative stress mediated by cobalt ferrite (CoFe2 O4) nanoparticles in zebrafish larvae." Environmental Toxicology, WILEY ONLINE LIBRARY, 31(12), 2068-2080.
[4] Boelen, A. (2009), "Thyroid hormones and glucose metabolism: the story begins before birth." Experimental Physiology, THE PHYSIOLOGIOCAL SOCIETY, 94(10), 1050-1051.
[5] Buha. A., Matovic, V., Antonijevic, B., Bulat, Z., Curcic, M., Renieri, E.A., Tsatsakis, A.M., Schweitzer, A., Wallace, D. (2018), "Overview of cadmium thyroid disrupting effects and mechanisms." International Journal of Molecular Sciences, MDPI, 19(5), 1501.
[6] Chatterjee, A. and Chatterji, U. (2010), "Arsenic abrogates the estrogen-signaling pathway in the rat uterus". Reproductive Biology and Endocrinology, BMC, 8(1), 80.
[7] Chatterjee, A. and Chatterji, U. (2011), "All-trans retinoic acid protects against arsenic-induced uterine toxicity in female Sprague-Dawley rats." Toxicology and Applied Pharmacology, ELSEVIER, 257(2), 250-63.
[8] Chatterjee, A. and Chatterji, U. (2017), "All-trans retinoic acid ameliorates arsenic induced oxidative stress and apoptosis in the rat uterus by modulating MAPK signaling proteins." Journal of Cellular Biochemistry, WILEY ONLINE LIBRARY, 118(11), 3796-3809.
[9] Choudhury, S ., Gupta, P., Ghosh, S., Mukherjee, S., Chakraborty, P., Chatterji, U., Chattopadhyay, S. (2016), "Arsenic-induced dose-dependent modulation of the NF-κB/IL-6 axis in thymocytes triggers differential immune responses." Toxicology, ELSEVIER, 357-358, 85-96.
[10] Claiborne, A. (1985), "Catalase activity." Boca Raton FL: CRC Press, 1, 283-284.
[11] Cremaschi, G.A., Gorelik, G., Klecha, A.J., Lysionek, A.E., Genaro, A.M. (2000), "Chronic stress influences the immune system through the thyroid axis." Life Sciences, ELSEVIER, 67(26), 3171-3179.
[12] Davey, J.C., Nomikos, A.P., Wungjiranirun, W., Sherman, J.R., Ingram, L., Batki. C.,

Lariviere, J.P., Hamilton, J.W. (2008), "Arsenic as an endocrine disruptor: arsenic disrupts retinoic acid receptor -and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis." Environmental Health Perspectives, NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES, 116(2), 165-172.
[13] Delgado,J.M., Dufour, L., Grimaldo, J.I., Carrizales, L., Rodríguez, V.W., Jiménez-Capdeville, M.E. (2000), "Effects of arsenite on central monoamines and plasmatic levels of adrenocorticotropic hormone (ACTH) in mice." Toxicology Letters, ELSEVIER, 117(1-2), 61-67.
[14] Elobeid, M.A. and Allison, D.B. (2008), "Putative environmental-endocrine disruptors and obesity: a review." Current Opinion in Endocrinology, Diabetes and Obesity, LIPPINCOTT, 15(5), 403-408.
[15] Frick, L.R., Rapanelli, M., Bussmann, U.A., Arcos, M.L.B., Genaro, A.M., Cremaschi, G.A. (2009), "Involvement of Thyroid Hormones in the Alterations of T-Cell Immunity and Tumor Progression Induced by Chronic Stress." Biological Psychiatry, ELSEVIER, 65(11), 935-942.
[16] Ghyselinck, N.B. and Duester, G. (2019), "Retinoic acid signaling pathways."Development, THE COMPANY OF BIOLOGISTS, 146(13), dev167502.
[17] Grau-Perez, M., Navas-Acien, A., Galan-Chilet, I., Briongos-Figuero, L.S., Morchon-Simon, D., Bermúdez, J.D., Crainiceanu, C.M., (2018), "Arsenic exposure, diabetes-related genes and diabetes prevalence in a general population from Spain." Environmental Pollution, ELSEVIER, 235, 948-955.
[18] Harvey, P.W. and Sutcliffe, C. (2010), "Adrenocortical hypertrophy: establishing cause and toxicological significance." Journal of Applied Toxicology, WILEY ANALYTICAL SCIENCE, 30(7), 617-26.
[19] Hassid, W.Z. and Abraham, S. (1957), "Chemical procedures for analysis of polysaccharides." Methods in enzymology, ACADEMIC PRESS, 3, 34-50.
[20] Jamal, Z., Das, J., Ghosh, S., Gupta, A., Chattopadhyay, S., Chatterji, U. (2019), "Arsenic-induced immunomodulatory effects disorient the survival-death interface by stabilizing the Hsp90/Beclin1 interaction." Chemosphere, ELSEVIER, 238, 124647.
[21] Jana, S ., Chattopadhyay, S., Dey, A., Perveen, H., Dolai, D. (2020), "Involvement of metallothionein, homocysteine and B-vitamins in the attenuation of arsenic-induced uterine disorders in response to the oral application of hydro-ethanolic extract of Moringa oleifera seed: a preliminary study." Drug and Chemical Toxicology, TAYLOR AND FRANCIS, 43(1), 1-12.
[22] Jollow, D.J., Mitchell, J.R., Zampaglione, N., Gillette, J.R. (1974), "Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite." Pharmacology, KARGER, 11(3), 151-169.
[23] Kinlein, S.A., Wilson, C.D., Karatsoreos, I.N. (2015), "Dysregulated hypothalamic–pituitary–adrenal axis function contributes to altered endocrine and neurobehavioral responses to acute stress." Frontiers in Psychiatry, FRONTIERS, 6: 31.
[24] Kulshrestha, A., Jarouliya, U., Prasad, G.B.K.S., Flora, S.J.S., Bisen, P.S. (2014), "Arsenic-induced abnormalities in glucose metabolism: Biochemical basis and potential therapeutic and nutritional interventions." World Journal of Translational Medicine, BAISHIDENG PUBLISHISNG GROUP, 3 (2), 96-111.
[25] Lam. (2020), "Cortisol, insulin, thyroid and the bioenergetic circuit." Food and Health, https://www.farmingportal.co.za/index.php/farminglifestyle/food-and-health/2119-cortisol-insulin-thyroid-and-the-bioenergetic-circuit.
[26] Linehan, C., Gupta, S., Samali, A., O'Connor, L. (2012), "Bisphenol A-mediated suppression of LPL gene expression inhibits triglyceride accumulation during adipogenic differentiation of human adult stem cells." PLoS One, PLOS, 7, e36109.
[27] Ling, S ., Shan, Q., Liu, P., Feng, T., Zhang, X., Xiang, P., Chen, K. (2017), "Metformin ameliorates arsenic trioxide hepatotoxicity via inhibiting mitochondrial complex I." Cell Death and Disease, NATURE, 8, e3159.
[28] Long, C.N.H., Katzin, B., Fry, E.G. (1940), "The adrenal cortex and carbohydrate metabolism." Endocrinology, OXFORD ACADEMIC, 26(2), 309-344.
[29] Madrigal, J.M., Ricardo, A.C., Persky, V., Turyk, M. (2019), "Associations between blood cadmium concentration and kidney function in the U.S. population: Impact of sex, diabetes and hypertension."Environmental Research, ELSEVIER, 169, 180-188.
[30] Marklund, S. and Marklund, G. (1974), "Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase." European Journal of Biochemistry, FEBS PRESS, 47(3), 469-474.
[31] Maronpot, R.R., Boorman, G.A., Gaul, B.W. (1999), "Pathology of the Mouse: Reference and Atlas Hardcover." Cache River Press Science, SAGE JOURNAL, 27(6), 699.
[32] Marrocco, I., Altieri, F., Peluso, I. (2017), "Measurement and clinical significance of biomarkers of oxidative stress in humans." Oxidative Medicine and Cellular Longevity, HINDAWI, 2017, 6501046.
[33] McGaugh, J.L. and Roozendaal, B. (2002), "Role of adrenal stress hormones in forming lasting memories in brain." Current Opinion of Neurobiology, ELSEVIER, 12(2), 205-210.
[34] Oh, H.C., Kwon, C.I., Hajj, I.I.E.I., Easler, J.J., Watkins, J., Fogel, E.L., McHenry, L., Sherman, S., Zimmerman, M.K., Lehman, G.A. (2017), "Low Serum Pancreatic Amylase and Lipase Values Are Simple and Useful Predictors to Diagnose Chronic Pancreatitis." Gut and Liver, NATIONAL LIBRARY OF MEDICINE, 11(6), 878-883.
[35] Pawlikowski, B., Wragge, J., Siegenthaler, J.A. (2019), "Retinoic acid signaling in vascular development." Genesis, WILEY ONLINE LIBRARY, 57(7-8), e23287.
[36] Sabir, S ., Akash, M.S.H., Fiayyaz, F., Saleem, U., Mehmood, M.H., Rehman, K. (2019), "Role of cadmium and arsenic as endocrine disruptors in the metabolism of carbohydrates. Inserting the association into perspectives." Biomedicine Pharmacotherapy, ELSEVIER, 114, 108802.
[37] Siddikuzzaman, C. Guruvayoorappan. and Berlin Grace, V.M. (2011), "All-trans retinoic acid and cancer." Immunopharmacology and Immunotoxicology, TAYLOR AND FRANCIS, 33(2), 241-9.
[38] Singh, M.K ., Dwivedi, S., Yadav, S.S., Sharma, P., Khattri, S. (2014), "Arsenic-induced hepatic toxicity and its attenuation by fruit extract of Emblica Officinalis (Amla) in mice. " Indian Journal of Clinical Biochemistry, SPRINGER, 29, 29-37.
[39] Stroth, N.and Eiden, L.E. (2010), "Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on pituitary adenylate cyclase-activating polypeptide signaling." Neuroscience, ELSEVIER, 165(4), 1025-1030.
[40] Sun, H.J., Xiang, P., Luo, J., Hong, H., Lin, H., Li, HB., Ma, L.Q. (2016), "Mechanisms of arsenic disruption on gonadal, adrenal and thyroid endocrine systems in humans. A review." Environment International, ELSEVIER, 95, 61-68.
[41] Sun, H.J., Li, S.W., Li, C., Wang, W.Q., Li, H.B., Ma, L.Q. (2017), "Thyrotoxicity of arsenate and arsenite on juvenile mice at organism, sub cellular and gene levels under low exposure." Chemosphere, ELSEVIER, 186, 580-587.
[42] Thang, N.Q ., Huy, B.T., Tan, L.V., Phuong, N.T.K. (2017), "Lead and arsenic accumulation and its effects on plasma cortisol levels in Oreochromis sp. "Bulletin of Environmental Contamination and Toxicology, SPRINGER, 99(2), 187-193.
[43] Trinder, P . (1969), "Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen." Journal of Clinical Pathology, BMJ JOURNAL, 22(2), 158-61.
[44] Tsigos,C . and Chrousos, G.P. (2002), "Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress." Journal of Psychosomatic Research, ELSEVIER, 53(4), 865-71.
[45] Ventura-Lima, J., Bogo, M.R., Monserrat, J.M. (2011), "Arsenic toxicity in mammals and aquatic animals: A comparative biochemical approach." Ecotoxicology and Environmental Safety, ELSEVIER, 74(3), 211-218.
[46] Walter, K.N., Corwin, E.J., Ulbrecht, J., Demers, L.M., Bennett, J.M., Whetzel, C.A., Klein, L.C. (2012), "Elevated thyroid stimulating hormone is associated with elevated cortisol in healthy young men and women." Thyroid Research, SPRINGER, 5(1), 13.
[47] Wei, Q. and Dong, Z. (2020), "The yin and yang of retinoic acid signaling in kidney diseases." The journal of Clinical Investigation, JCI, 130(10), 5124-5126.
[48] Wondisford, F.E. (2015), "A direct role for thyroid hormone in development of the adrenal cortex." Endocrinology, OXFORD ACADEMIC, 156(6),1939-1940.
[49] Wu, J., Wan, X., Zhang, H., Li, W., Ma, M., Pan, B., Liang. X., Cao, C. (2019), "Retinoic acid attenuates contrast-induced acute kidney injury in a miniature pig model." Biochemical and Biophysical Research Communications, ELSEVIER, 512(2), 163-169.