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RESEARCH ARTICLE

Enzymatic Implications and Biochemical Assessments of Doxorubicin (DXR) in TCA-induced Neoplasia Wistar Rats

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ABSTRACT

Despite decades of attempt to curb the menace of cancer and the analogous chemotherapy, the disease remains one of the most dreadful with high annual mortality rate. Trichloroacetic acid (TCA) is a micro-contaminant of groundwater with carcinogenic effect. The effect of relatively low dose of doxorubicin (DXR) was examined in the body chemistry of early and late TCA cancer induced Wistar rats; the internal tissues were used to assay for glucose, albumin, total protein, peptidase, ALT, LDH, ACP and ALP. TCA induced multineoplasia in the tested rats and this was characterized with tissues atrophy as manifested in the elevated level of serum protein and peptidase activity; similar to the adopted treatments but seemed to have positively modulated the liver total protein. Serum and liver glucose levels remained unaffected. Oral gavage of TCA caused hypoalbuminemia and hyperalbuminemia in the serum and kidney, but there was no significant effect ($P>0.05$) in the heart. Generally, adopted treatments stimulated peptidase activity in serum with high level of protein and albumin in serum and kidney respectively. There was no significant alteration in ACP and ALP. ALT, which appeared to be elevated and reduced in the liver and serum respectively, was moderated by the adopted treatments. Likewise, the elevated LDH activity was reduced by the treatments but its activity was extremely insignificant in the kidney. Therefore, irrepressible consumption of TCA contaminated water may result into neoplasia. DXR, at relatively low dose, may be useful in the management of cancer without causing insufferable physiological changes.

KEYWORDS

Cancer, Doxorubicin, TCA, Enzymes, Proteins, Glucose

INTRODUCTION

Cancers, in most cases, are terminal diseases distinguished with the formation of unregulated and uncontrollable cell growth (neoplasm) with disproportionate biological functions.[1]

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Metastasis is a characteristic phenomenon that differentiates malignant from benign cancers and responsible for high mortality rate.[2] Medically, cancer chemotherapy is best targeted at the point of metastasis. Generally, cancer has been proposed to be caused by several factors such as chemicals (carcinogens),[3] hereditary,[4] exposure to radiation (ionizing and non-ionizing),[5] diets,[6] asbestos dusts (occupation),[7] abnormalities in hormones,[8] oncogene and tumour suppressor gene activities;[9] alcohols,[10]

cigarettes[11] and infections.[12] Trichloroacetic acid (TCA) is currently a compound of considerable interest to the scientific and regulatory communities as a potential human carcinogen. TCA and dichloroacetic acid (DCA) are the major haloacetic acids (HAA) found in public drinking water supplies.[13] HAA and certain other disinfectant byproducts are formed by reaction of chlorine with organic compounds present in water. TCA is also a major metabolite of halocarbon solvents such as trichloroethylene (TCE) and perchloroethylene (PCE), which are common drinking water contaminants of industrialized and waste site groundwater.[14] Experimentally, the three can cause liver cancer in mice but not in rats.[15] However, cancer is mostly considered a preventable disease.[16] Hypothetically, cancer risk can be reduced by avoiding cancer-causing agents and the habitual consumption of functional foods for preventive purpose.[17] Certain fortified drugs that contain antioxidants, unsaturated fatty acids, vitamins,[18] hormones and vaccines[19] are now adopted in cancer chemopreventive therapy.[20]

Invading neoplasia requires systemic chemotherapy with anticancer drugs to control metastases.[21] Hitherto, anticancer drugs, like actinomycin A, were effectively obtained from bacteria, while others were from plants and fungi.[22] Subsequently, a variety of antitumour antibiotics (including doxorubicin (DXR), daunorubicin, actinomycin D, mitomycin C and bleomycin) have been isolated by fermentation from various *Streptomyces* species.[23] The notion that these compounds had unique structural features with antitumour activity initiated analogue research but only few have survived the test of time. Doxorubicin hydrochloride is a potent antitumor agent that is used, sometimes in combination with other drugs,[24] to treat various types of human cancer such as carcinomas, sarcomas and lymphomas of both solid and liquid tumours.[25] The antitumor activity of DXR, an anthracycline antibiotic, is mediated by its ability to stimulate the formation of a variety of free radical species with the ability to intercalate DNA and inhibit topoisomerase II.[26] Although, doxorubicin-induced cardiac, nephritic and hepatic injuries appear to be multi-factorial, a common phenomenon is the cellular damage mediated by reactive oxygen species (ROS).[27]

Regarding the toxicity of DXR, though Imondi reported that 15-25mg/kg dose range of DXR is usually adopted in humans and laboratory animals,[28] however cumulative dose of DXR has led to hepatotoxicity,[29] nephrotoxicity[30] and cardiotoxicity;[31] including congestive heart failure, dilated cardiomyopathy, systolic dysfunction[32] and death as reported by Singh.[33] These effects are mainly due to the molecular structure of this drug, which can generate radicals after metabolism.[34] In turn, the radicals act as catalyst for peroxidation, membrane rupturing, infarction, toxicity and necrosis;[35] and are manifested in the biochemical alteration of clinical enzymes such as LDH, ALT, ACP, ALP, CK, AST; body electrolytes and physiologic molecules.

Clinically, DXR elevates serum level of LDH and CK.[36] Hepatotoxicity study by Osama[29] reported elevation in the serum levels of ALT, AST, ALP, GGT and total bilirubin at a cumulative dose of 24mg/kg in 6 weeks.[37] However, reduced hepatic glutathione transferase, peroxidase, SOD, CAT activities with high lipid peroxidation was reported. Acute adverse effects of doxorubicin can include nausea, vomiting, heart arrhythmias, typhlitis, neutropenia, alopecia, urine discoloration, infarctions and cardiomyopathy.[38] With these effects, many enzymes and biomolecules of clinical interest are exuded into the blood beyond threshold concentrations. This invariably leads to complication and death, which are usually experienced by cancer sufferers placed on prolong DXR chemotherapy.

Following the high rate of underground water consumptions with micro contamination of TCA and the alarming incidence of cancers, then there is need for correlation studies to predict the occurrence of cancer in both short- and long-term consumptions of such water *vis-à-vis* the management of such disease with drug of choice. Using biochemical and physiological

biomarkers, this work assessed the implications of DXR, as one of the potent antitumor drug, in TCA-induced neoplasia Wistar rats.

MATERIALS AND METHOD

Experimental Animals

Twenty-eight Wistar strain rats were obtained from the Animal Breeding Unit of the College of Medicine of the University of Lagos, Akoka Lagos State Nigeria. The animals, weighed 200 ± 10g, were maintained in 12 hour light: 12 hour dark at controlled temperature (25 ± 2^{o}C) and humidity (60 ± 5%) and kept in the animal house of the Department of Biochemistry, Lagos State University, Ojo, Lagos State Nigeria.

The animals were allowed to acclimatize for one week in a plastic cage (2ft. x 2ft.) at seven per cage (4 females and 3 males). This was to allow optimal interactions. Certified commercial feed and water were given *ad libitum*.

All animals were treated in accordance with the recommendations of the Ethical Committee on the Use and Management of Laboratory Animals, College of Medicine, University of Lagos, Nigeria as adopted from the Institute for Laboratory Animal Research Guides for the Care and Use of Laboratory Animals (ILAR, 2011).

Animal Grouping

The experimental animals were divided into four groups as followed:

"A" as Control;

"B" (TCA-induced cancer) as Cancer;

"C" (TCA-induced cancer + Doxorubicin) as TCA+DXR (early induced) and

"D" (Doxorubicin + TCA-induced cancer) as DXR+TCA (late induced).

Chemicals

Trichloroacetic acid (99.7% purity) was purchased from Sigma-Aldrich, USA, NaOH pellet from BDH, doxorubicin hydrochloride ((8S,10S)-10-[(3-Amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)-oxy]-8-glycoloyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride, MF = $C_{27}H_{29}NO_{11}$•HCl, MW = 579.99g/mol) powder for injection and physiological saline infusion were purchased from Pharmaceutical outlet within the suburb.

Each bottle supplied by the manufacturer contained 10mg of lyophilized DXR.

Methods

Preparation of Trichloroacetic Acid[39]

One hundred ml of trichloroacetic acid (TCA) solution at concentration of 500mg/ml, which was previously neutralized with 1M NaOH solution to a resultant pH of 6.3, was prepared.

$$CCl_3COOH_{(aq)} + NaOH_{(aq)} \longrightarrow CCl_3COONa_{(aq)} + H_2O_{(l)}$$

Formulation of Doxorubicin Injection

Doxorubicin hydrochloride powder for injection was dissolved with 5ml of 0.9% NaCl solution at a concentration of 2.0mg/ml.

Experimental Design

After one week of acclimatization, the **control** group was administered 0.5ml physiological saline by intraperitoneal route (IP) daily for 30 days.

The **cancer-induced** group received 1ml/kg body weight (b.w) of neutralized TCA solution for five consecutive days, by oral gavage.

TCA-DXR group received 1ml/kg body weight of neutralized TCA solution for five consecutive days, by oral gavage.

Seventy-two hours after, they were administered doxorubicin at a concentration of 2mg/kg b.w by intraperitoneal route. DXR was given once a week for 4 weeks; a cumulative dosage of 8mg/kg b.w.

Conversely, **DXR-TCA** group received a cumulative dose of 8mg/kg b.w doxorubicin in 4 weeks. After 72 hours of the last administration, 1ml/kg b.w of neutralized TCA solution was orally given for five consecutive days.

Animal Sacrifice

After the completion of respective dosages, animals were allowed to fast overnight and anaesthetized in a desiccator pre-soaked with diethylether (DEE). All animals were sacrificed by cervical decapitation. During this study, no mortality was recorded.

Collection of Blood Samples

Blood samples were collected by cardiac puncture using 5ml hypodermic needle and heparinised syringe. The blood was collected in a sample bottle and allowed to clot for 15 minutes. The blood sample was centrifuged at 3000rpm for 5 minutes and stored in a refrigerator at 4^{o}C. All sera were used within 48 hours.

Dissection of Internal Tissues

The animals were further dissected. The liver, kidney and heart were carefully excised and washed with saline solution to remove the bloodstains. 500mg of each of the tissues was homogenized in 5ml ice-cold deionized distilled water. The homogenates were kept in the refrigerator at 4^{o}C and were used within 48 hours.

Biochemical Assays

Total protein, glucose content and alanine aminotransferase (ALT) were determined in the liver homogenate and serum. Total albumin was quantified in the kidney, heart and serum. Proteolytic activity of peptidase was determined in the kidney and serum.

Lactate dehydrogenase (LDH) was assayed in the liver, kidney and serum. Acid phosphatase (ACP) and alkaline phosphatase (ALP) were assayed in the heart and serum. All assays were carried out using RANDOX Laboratory Commercial Kits.

Statistical Analysis[40]

The presented data were expressed as mean ± SEM. Statistical significance was examined using both one-way and two-way analysis of variance (ANOVA). Mean differences were considered statistically significant at $P<0.05$. All values were read in triplicate.

RESULTS AND DISCUSSION

Tumour Formation

There were remarkable formations of multiple hard tumours in all the TCA-induced cancer groups. The tumours of different sizes were randomly located within and outside the intestinal lumen. There were formations of enlarged perforated tissues. Dissection of these isolated hard tissues showed yellowish-white cristae with patches of fatty layers underlying the dermis of the tissue. However, there was no any noticeable tumour grown in early- or late-induced DXR treated groups, though the rats appeared dull and insipid.

Trichloroacetic acid (TCA), a micro-contaminant of groundwater, has been reported as hepatocarcinoma agent especially in mice.[15] In addition, TCA was not only confirmed as potential carcinogenic compound in this study, it was also capable of inducing numbers of isolated tumours within the visceral tissues of Wistar rats.

With reference to the work of Abdel-Hamid[41], among other researchers, who induced liver cancer with TCA solution, hepatocarcinoma was not only induced in this study but also series of hard polyps of different sizes dispensed in both upper and lower abdominal cavities of the rats. The word “multineoplasia” was conscientiously used to describe the formation of these polyps.

Careful dissection and examination of this tumour with painstaking comparison confirmed the formation of multiple neoplasia. Serendipitously, there was no any noticeable appearance of polyp of any kind in both early- and late-TCA induced cancer DXR treated groups.

By the design used in this work, thorough clinical analysis and yardstick comparison showed that synergistic dosage of DXR and TCA, in any other of administration, was not able to cause any noticeable toxicity in the heart, kidney and liver.

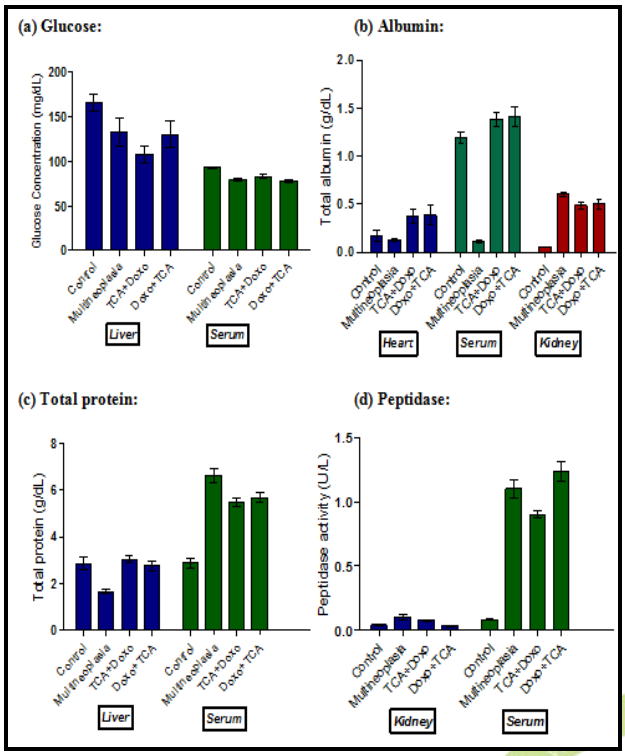

Figure: 1 Glucose, albumin, total protein and peptidase activity in the liver, heart, kidney and serum of the tested rats; (Each bar is presented as mean ± SEM, *n =3*)

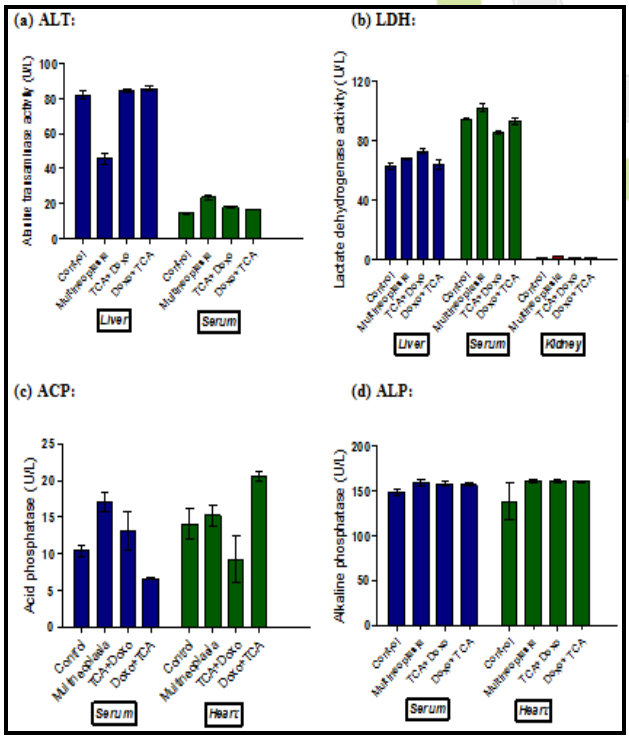

Figure: 2 Activities of ALT, LDH, ACP and ALP activities in the liver, heart, kidney and serum of the tested rats; (Each bar is presented as mean ± SEM, *n =3*)

Glucose, Albumin, Total Protein and Peptidase (EC: 3.4.11)

The results presented in this work have shown some significant alterations in the body chemistry of the tested rats because of the oral administration of TCA. The glucose level as observed in the liver within the positive control, the induced and the late induced cancer DXR treated groups appeared not to be significantly different from each other but higher *(P<0.01)* in control compared to the early-induced DXR treated group (Figure 1a). There was no statistical different in the level of serum glucose as observed in all the groups. Careful comparison of glucose levels between the liver and the serum showed statistical differences in the controls *(P<0.001)*, induced and late induced groups *(P<0.01)*. There was no statistical different in the early induced groups. The present data have shown that induction of cancer with TCA together with its treatment, whether early or late, may not have significant effect on both serum and liver glucose levels and that the data supported the liver as the sole storage of glucose.

Figure 1b shows the level of albumin in the heart, serum and kidney. Generally, low level of albumin was recorded in the heart and kidney as compared to the serum. In the heart, there was no any noticeable difference in the level of albumin in all the observed groups unlike the kidney, which showed lowest albumin level (0.052±0.003g/dL) in the control as compared to others. However, the albumin level was significantly lower (0.11±0.02g/dL) in the cancer-induced group than any other group in the serum. Two-way analysis of variance showed significant higher level *(P<0.001)* of albumin in the serum against kidney and the heart, though there was an exception in the cancer induced groups of the heart and serum, where there was no difference. In the heart and the kidney, there was no statistical different in the level of albumin in all the observed groups except in the cancer group where higher level of albumin was recorded in the heart (0.12±0.02g/dL) against the kidney (0.052±0.003g/dL). Each of the compared group

of serum was constantly higher than there corresponding group in the kidney.

The total protein in the liver and serum is shown in Figure 1c. In the liver, there was no significant different *(P>0.05)* in the level of total protein in the control, early and late induced cancer DXR treated groups but the level of total protein in the multineoplasia group was significantly lower *(P<0.01)* than the control (1.65±0.1g/dL vs. 2.86±0.3g/dL respectively) and invariably than any other group. In the serum, all the induced groups constantly had higher level of total protein than the control group (2.87±0.21U/L, *P<0.001*). Consistently, the level of total proteins was quite high in the serum and significantly different *(P<0.001)* from the liver except for their controls.

Peptidase activity is shown in Figure 1d. In the kidney, the activity of this enzyme remained fairly the same in all the studied groups but in the serum all the induced groups have elevated level of the activity of this enzyme with high significant different *(P<0.001)* as compared to the control. There was no different in the level of the activity of this enzyme in both controls (kidney, 0.042±0.005U/L vs. serum, 0.08±0.002U/L). However, the activity of this enzyme was constantly higher in the serum than the kidney when comparing the corresponding induced groups.

Administration of TCA was marked with reduced serum albumin and total protein in the liver; these are conditions described as hypoalbuminemia and hypoproteinemia respectively, but hyperalbuminemia was witnessed in the kidney except in the control group. In the work of Lumpkin,[42] it was discovered that plasma proteins have high affinity for TCA in other to reduce the TCA uptake by the liver and other peripheral tissues, the reason why unbound albumin was low in the serum but high in the kidney, because of blood filtration. Relatively high pumping and metabolic rate of the cardiac muscles may not have allowed the levels of TCA and/or DXR, as observed in this work, to have any significant effects. In addition, either TCA or TCA-DXR administration persistently caused serum hyperproteinemia and increased activity of peptidase (Figure 1c). This was a retroactive tandem between the levels of total protein and peptidase in the serum. High level of total protein led to high activity of protein hydrolase (peptidase). This was an indication of the occurrence of tissues atrophy and these were manifested as dullness and insipidness as symptoms in the rats. Meanwhile, the adopted treatments (TCA+DXR and DXR+TCA) positively modulated this flux in the liver. There was no prominent activity of peptidase in the kidney.

Alanine Transaminase (ALT) EC: 2.6.1.2 and Lactate Dehydrogenase (LDH) EC 1.1.1.27

The activity of alanine transaminase (ALT) is shown in Figure 2a. There was no significant different in the level of the activity of this enzyme in the liver in all the observed groups except in the cancer induced group (multineoplasia) where a significant reduced *(P<0.001)* activity of 45.82±3.24U/L was observed as compared to the control (82.2±1.97U/L). Contrarily, a higher activity *(P<0.01)* of ALT was recorded in the serum of TCA-induced cancer group (23.48±1.7U/L) as compared with the control (14.41±0.23U/L). This value was also significantly higher comparing with the remaining groups (TCA+DXR, early induced and DXR+TCA, late induced). Significantly, higher ALT activity was recorded in the liver than the serum in all the corresponding groups studied *(P<0.001)*.

Figure 2b shows the activity of lactate dehydrogenase (LDH) in the liver, serum and kidney. Each of the group considered in the serum showed significant different *(P<0.001)* when compared with their corresponding group in the liver and kidney. Highest and lowest activities of LDH were recorded in the serum and kidney respectively. There was no significant different *(P>0.05)* in the activity of this enzyme within all the groups in the kidney. Statistically, the activity of this enzyme was significantly higher in the multineoplasia (liver,

85.29±1.10U/L) and (serum, 72.91±1.75U/L) groups than their corresponding control (liver, 94.35±0.48I/U, *P<0.1*) and (serum, 62.54±2.22I/U, *P<0.01)* respectively but the adopted treatment positively regulate this flux.

Sequel to the earlier impression that random treatment using TCA-DXR, as designed in this study, may not result into any toxicity in the liver, kidney and heart; TCA administration drastically reduced the activity of ALT in the liver but was pre-empted by DXR treatment. Similarly, higher activity of this enzyme noted in the serum was again moderated by DXR, either in the early or late treatment. The activity of LDH was extremely inconspicuous in the kidney in all the groups. Ordinarily LDH activity is usually minimized, unless in a disease/hypoxia state. Nevertheless, the activity of LDH in the liver and serum may further buttress the non-toxicity of the regimen used in this work.

The activity of lactate dehydrogenase (LDH), an anaerobic constitutive enzyme responsible for the conversion of pyruvate to lactate in the peripheral tissues, was significantly higher in the multineoplasia but was regulated following the DXR treatment, both in the liver and serum. Supportably, Verma and Vinayak have reported increased LDH activity in various tissues as well as serum of TCA-treated rats.[43] Because LDH is used as a biomarker of cancer,[44] the progressive significant decrease in the activity of LDH during tumour development by low dose treatment of DXR indicated anticarcinogenic action of this drug, the mechanism by which anaerobic metabolism of tumour cells is downregulated.

Acid (EC 3.1.3.2) and Alkaline Phosphatases (EC 3.1.3.1)

Both acid phosphatase (ACP) and alkaline phosphatase (ALP) as shown in Figure 2c and d depicted similar activities. There was no significant different in the activities of ACP and ALP within all the considered groups, both in the serum and the heart. Similarly, comparing the activity of ALP in the serum with the heart within their corresponding groups showed no significant different *(P>0.05)*. ACP nearly showed the same but for DXR+TCA group in the heart that has higher activity of ACP (0.21±0.007U/L) than its corresponding serum group (0.07±0.002U/L).

In other to re-affirm the non-cardiotoxicity of the treatment adopted in this study, lysosomal enzymes (ACP and ALP) failed to be altered in their activities. Though these enzymes are indexes of prostate cancer,[45] the present report has not confirmed the occurrence of such in all the induced groups. This might probably be the reason why lysosomal enzymes appeared not to be affected by TCA administration in the heart and the serum. However, late induction of cancer in this work appeared to play a significant role in elevating the activity of acid phosphatase in the heart. This deserves more insight.

Thus, the implications of TCA administration appeared to be more deleterious in the tissues and this was indirectly shown in the serum, where all exudates resided. TCA was able to induce higher level of albumin in the kidney and elevated activity of peptidase, LDH and ALT in the serum than their controls. Contrarily, oral gavage of TCA resulted in reduced activity of ALT in the liver and lower level of total protein and albumin in the liver and serum respectively.

CONCLUSION

Therefore, TCA was confirmed as carcinogenic substance and that DXR at relatively low dose may be medically important for cancer treatment, management and prevention. Though the treatment adopted yielded high protein turnover, an indication of tissues atrophy nonetheless, the template of this work still supported the use of DXR, at relatively low dose and that its advantage may possibly outweighed disadvantage.

Regulation and proper precaution may be suggested to checkmate the level of contamination of TCA and its adjuncts in all domestic groundwater usage.[14] Nevertheless, further clinical trials are therefore recommended for confirmation.

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