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The correlation between severe complications and blood group types in COVID-19 patients; with possible role of T polyagglutination inpromoting thrombotic tendencies

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ABSTRACT

Introduction: Coronavirus disease-19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is still posing detrimental effects on people. An association between contracting COVID-19 and the ABO blood group type has been determined. However, factors that determine the severity of COVID-19 are not yet fully understood. Thus, the current study aimed to investigate whether the ABO blood group type has a role in the severity of complications due to COVID-19. Materials and methods: Eighty-Six ICU-admitted COVID-19 patients and 80 matched healthy controls were recruited in the study from Baish general hospital, Saudi Arabia. ABO blood grouping, complete blood count (CBC), CBC-derived inflammatory markers, coagulation profile, D-Dimer and anti-T antigen were reported. Results: Our data showed that patients with blood groups O and B are more protective against severe complications from COVID-19, as compared to patients with blood groups A and AB. This could be partially attributed to the presence of anti-T in blood group A individuals, compared to non-blood group A. Conclusion: The current study reports an association between the ABO blood group and the susceptibility to severe complications from COVID-19, with a possible role of anti-T in driving the mechanism of the thrombotic tendency, as it was also correlated with an elevation in D-dimer levels.

Keywords: COVID-19; ABO blood group; D-dimer; anti-T; severity

1. Introduction

The rapid spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), at the end of 2019, still exhibits a huge burden and challenges on healthcare systems and economies including testing resources and patients' management. The novel coronavirus disease 2019 (COVID-19) might lead to

severe acute respiratory syndrome and has shown association with increased morbidity and mortality especially among elderly patients and patients with commodities such as diabetes, hyperten sion, obesity, and cardiovascular diseases [1–3]. In addition, COVID-19 has several non-respiratory complications such as systematic complications including thrombosis, septic shock, and disseminated intravascular coagulopathy (DIC) [4].

Studies have suggested that ABO blood group system is not only a potential risk factor for the susceptibility and severity of COVID-19 [5,6], but also for (i) viral infections such as Hepatitis B virus, Middle Eastern respiratory syndrome coronavirus (MERS) and severe acute respiratory syndrome coronavirus (SARS) [6,7] and (ii) other non-viral diseases such as myocardial infarction, cancer, acute renal injury, and venous thromboembolism [7,8].

The current study aimed to (i) determine and correlate the ABO blood group type with D-dimer levels among intensive care unit (ICU)-admitted COVID-19 patients, and (ii) correlate the ABO blood group type with the T polyagglutination in Jazan city, Saudi Arabia.

2. Materials and methods

The current study was a case-control study, which involved a total of 86 ICU-admitted COVID 19 patients (42 males and 44 females) and 92 healthy individuals (47 males and 45 females). The patients and controls were recruited from Baish General Hospital, Jazan region, Saudi Arabia. All COVID-19 patients were confirmed positive for SARS-COV-2 using RT-polymerase chain reaction (RT-PCR). The ICU admission of the patients was based on the Saudi Arabia ICU-admission criteria [9].

2.1. Sample collection

Venous blood samples were collected from patients and control in ethylenediaminetetraacetic acid (EDTA) and sodium citrate anticoagulated tubes. The EDTA tube was used for determining com plete blood count (CBC) and ABO blood typing. The sodium citrate tube was used for coagulation profile and D-dimer analysis.

2.2. Complete blood count analysis

The CBC was obtained by analyzing the EDTA tube using Sysmex XN–1000 Hematology Ana lyzer (Kobe, Japan).

2.3. Complete blood count-derived inflammatory markers

The CBC-derived inflammatory markers, including neutrophil/lymphocyte ratio (NLR), plate let/lymphocyte ratio (PLR), and platelet/neutrophil ratio (PNR), were calculated as previously de scribed [10,11].

2.4. Coagulation profile and D-dimer measurement

Coagulation profile (prothrombin time [PT], activated partial thromboplastin [aPTT]) and D-di mer levels were measured using the Stago compact analyzer (Diagnostica Stago, Asnieres sur Seine, France).

2.5. ABO blood group typing

Red cell suspension prepared from samples collected from COVID-19 patients were reacted with antisera A & B and were observed for agglutination indicating the presence or absence of corresponding antigen. ABO blood group antisera were obtained from Crescent Diagnostics (Jeddah, KSA).

2.6. Anti-T testing

The anti-T test was performed by mixing one drop of anti-T (Arachis hypogaea lectin) with one drop of 3–5% suspension of the patient's RBCs. After 15 minutes of incubation at room temperature and centrifugation at 1000 rpm for one minute, agglutination was observed macroscopically indicating positive results.

2.7. Ethical approval

The study was approved by the Jazan Health Ethics Committee "Jazan IRB", Ministry of Health (project no. 2053) and carried out according to the Declaration of Helsinki.

2.8. Statistical analysis

GraphPad Prism version 9 (San Diego, USA) was used for the statistical analysis. A chi-squared test was performed to test non-parametric data, and student's unpaired t-test was applied for group comparison of parametric data. The CBC data were presented as mean ± standard deviation (SD). P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic data

Eighty-six ICU-admitted COVID-19 patients (42 males and 44 females; patient group) and ninety-two healthy controls (47 males and 45 females; control group) were recruited. The male to female ratio was almost 1:1 in both groups. The demographic data of the patients and control groups are shown in Table 1. There were no significant differences between the two groups in number, gender and age ($P > 0.05$).

Table 1. Complete blood count and CBC-derived inflammatory markers in the study cohort. Data are presented as mean ± SD. Unpaired student t-test was used to assess the significant differences between the patients and control groups.

Parameters	Patients	Controls	*P* value
	Mean ± SD	Mean ± SD	
Number (male/female)	86(42/44)	92(47/45)	>0.05
Age (years)	59.6 ± 18.5	55.1 ± 25.3	>0.05
WBCs ($\times 10^9$/L)	12.8 ± 10.3	6.6 ± 1.8	<0.0001
Neutrophils ($\times 10^9$/L)	9.9 ± 20.2	2.9 ± 1.3	0.0002
Lymphocytes ($\times 10^9$/L)	1.6 ± 1.4	2.7 ± 0.8	<0.0001
Monocytes ($\times 10^9$/L)	0.6 ± 0.9	0.6 ± 0.2	>0.05
Eosinophils ($\times 10^9$/L)	0.1 ± 0.1	0.3 ± 0.2	<0.0001
Basophils ($\times 10^9$/L)	0.05 ± 0.07	0.04 ± 0.02	>0.05
RBCs ($\times 10^{12}$/L)	3.9 ± 0.8	5.1 ± 0.5	<0.0001
Hemoglobin (g/dL)	10.6 ± 2.0	13.7 ± 1.9	<0.0001
Hematocrit (%)	33.5 ± 6.4	42.1 ± 5.8	<0.0001
MCV (fL)	84.9 ± 9.0	82.3 ± 6.6	0.0176
MCH (pg)	26.9 ± 3.3	26.9 ± 2.6	>0.05

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MCH (pg)	26.9 ± 3.3	26.9 ± 2.6	>0.05

MCHC (g/dL)	31.7 ± 1.8	32.6 ± 0.9	<0.0001
RDW (%)	15.2 ± 2.9	16.8 ± 1.9	<0.0001
Platelets ($\times 10^9$/L)	215 ± 115	286 ± 67	<0.0001
NLR	6.2 ± 5.5	1.1 ± 1.5	<0.0001
PLR	134 ± 215	104 ± 79	>0.05
PNR	21.7 ± 19	98 ± 51	>0.05

Note: WBCs: White blood cells; RBCs: Red blood cells; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean cell hemoglobin concentration; RDW: Red cell distribution width; NLR: Neutrophil/lymphocyte ratio; PLR: Platelet/lymphocyte ratio; PNR: Platelet/neutrophil ratio.

The overall CBC parameters showed an abnormal pattern in the patient group (Table 1). Patients had higher WBC, neutrophils ($P < 0.05$) and basophils ($P > 0.05$) counts as compared to the control group. The lymophcytes, eosinophils, ($P < 0.05$) and monocytes ($P > 0.05$) were low in patient group as compared to the control group. Furthermore, the RBCs, hemoglobin, hematocrit, red cell distribu tion width (RDW) and mean cell hemoglobin concentration (MCHC) were significantly low in the patient group as compared to control group ($P < 0.0001$; Table 1). On the other hand, the mean cell volume (MCV) was significantly high in patients as compared to control (Table 1).

3.2. Complete blood count-derived inflammatory markers

The inflammatory markers i.e., NLR ($P < 0.0001$) and PLR ($P > 0.05$) were high in patients compared to controls (Table 1). PNR values were less in patient group compared to control group ($P > 0.05$; Table 1).

3.3. Coagulation profile and D-dimer measurement

The coagulation profile tests (PT and aPTT) were markedly prolonged in the patient group as compared to the control group ($P < 0.0001$; Table 2). The D-dimer levels were elevated in patients(Table 2).

Table 2. Coagulation profile and D-dimer in the study cohort. Data are presented as mean ± SD. Unpaired student t-test was used to assess the significant differences between the patients and control groups.

Parameters	Patients	Controls	*P* value
	Mean ± SD	Mean ± SD	
Prothrombin time (seconds)	18.1 ± 7.7	12.5 ± 1.9	<0.0001
Activated partial thromboplastin time (seconds)	42.6 ± 12.8	31.7 ± 5.2	<0.0001
D-Dimer (ng/mL)	4.1 ± 6.5	NA	

Note: NA: Not available.

3.4. ABO blood group in the study cohort

The ABO group typing analysis revealed the following sequence A > O > B > AB in the patient group (Table 3). Out of 86 patients, 34 (39.5%) had blood group A, 22 (25.6%) blood group O, 17 (19.8%) blood group AB, and 13 (15.1%) patients had blood group B. On the other hand, in the control group the blood group is in 55 out of 92 representing 59.8%, followed by blood group A (28.3%), blood group B (10.9%) and blood group AB (1.1%).

Table 3. Distribution of ABO group systems in the study cohorts.

Blood group	Patients number (%)	Controls number (%)
O	22 (25.6)	55 (59.8)
A	34 (39.5)	26 (28.3)
B	13 (15.1)	10 (10.9)
AB	17 (19.8)	1 (1.1)
Total number	86 (100)	92 (100)

3.5. Anti-T analysis

The anti-T analysis was positive in 28 out the 86 patients and negative in 58 patients as well as healthy controls (Table 4).

Table 4. Anti-T in the studied population

Anti-T	Patients (n = 86)	Controls
Positive	28	0
Negative	58	92

3.6. Association of ABO blood grouping and anti-T analysis

Twelve patients out of the 34 with blood group A were anti-T positive, followed by 9 out of 22 patients with blood group O (Figure 1).

3.7. Association of ABO blood grouping vs D-dimer levels

The D-dimer levels were found to be more elevated in patients with blood group A. This elevation was twice the D-dimer levels found in blood O, B, and AB (Figure 1)

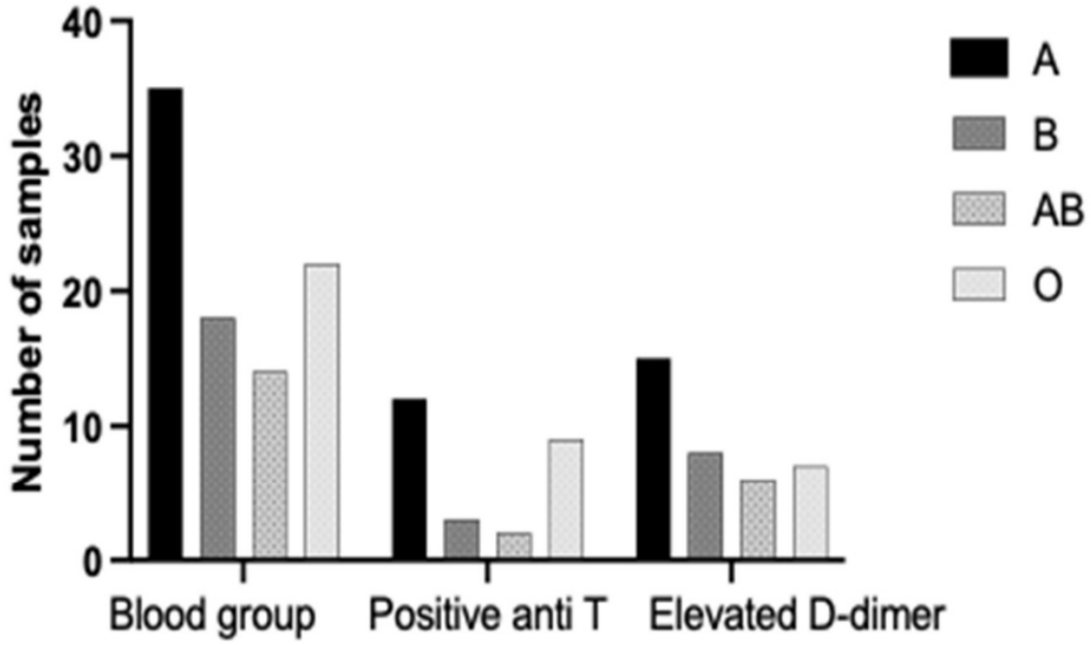

Figure 1. Distribution of ABO blood types, positive anti T antibodies and D-dimers levels in patients with COVID-19.

4. Discussion

The current study investigated the prevalence of ABO blood group system in ICU-admitted COVID-19 patients and correlated each ABO blood group system with other biochemical parameters including D-dimer, anti-T polyaggulintation.

The current study reported abnormalities in the CBC parameters, including increased WBC (leu kocytosis), which is a feature of COVID-19 infection. These finding are in agreement with previous reports [10–12]. The WBC count have been reported to be high in COVID-19 patients. In addition, several studies also reported high WBC count in severely, ICU, infected COVID-19 patients as com pared with mild infected COVID-19 patients [12]. Our data showed high neutrophil count (neutro philia), low count of lymphocytes, RBC count, and low hemoglobin concentration in the ICU-admitted COVID-19 patients as compared to the matched healthy controls. Indeed, neutrophilia and lymphopenia have been suggested to be associated with severity and mortality in COVID-19 patients [10,13,14].

Similarly, the inflammatory markers, NLR and PLR were found to be elevated and lower PNR in the patients cohort, similar to other reported studies [10,11]. The inflammatory response is a hallmark of COVID-19 infection and have been associated with the cytokine storm [15].

The current study showed that majority of the patients who were shifted to ICU due to COVID 19 were patients with blood group A (39.5%) followed by O (25.6%) as compared to those with other ABO blood group types (Figure 1). It is worth mentioning that O and A blood group types are most common blood group system among the Saudi population with frequencies of ~59% O and ~29% A in Jazan area respectively [16–18]. The above data are consistent with the previous studies suggesting a link between the susceptibility to SARS-CoV-2 infection and ABO blood group types [19–21]. A recent study by Muñoz-Diaz et al. (2021) reported a strong association between susceptibility to COVID-19 and the ABO blood group types [5]. Individuals with blood group A had higher risk of acquiring COVID-19 while those with blood group O had lower susceptibility [21]. Similarly, SARS has also been found to be more frequent in individuals with blood group A [22].

Furthermore, it has also been reported that the severity of COVID-19 and mortality risk, as well as the risk of hospitalization, are higher in patients with blood group A as compared to those with non blood group A [5]. Importantly, ICU admitted patients with blood group O were reported to be less susceptible to severe complications/manifestations of COVID-19, as indicated by current data and others [23]. Similar findings have also been observed among females with COVID-19 [24]. It was suggested that anti-A antibody, found in persons with blood group O or B, appears to antagonize the link between SARS-CoV-1 and the receptor for angiotensin-converting enzyme 2 (ACE2), which is expressed by the target cells of the host [25]. This could explain the reason behind the correlation between the ABO blood type and COVID-19 severity as shown in the current study.

It is reasonable to consider and address that ABO blood types may also be possible determinants of susceptibility to SARS-CoV-2 infection and its severity, as the virus, SARS-CoV-2, also binds to ACE2 [26,27]. Moreover, it has also been found that critically ill COVID-19 patients with blood group type A or AB are linked with an increased risk of requiring mechanical ventilation, continuous renal replacement therapy (CRRT), and prolonged stay in ICU, as compared to patients with blood group B or O [28]. However, further studies are required to identify the biological mechanisms underpinning these findings. Kibler et al. (2020) declared that patients with A blood group were especially prone to develop the disease and showed unfavorable outcomes [29]; which is consistent with the findings of our study. Furthermore, Zhao et al. (2020) found that the incidence, severity, and mortality of COVID 19 were more common in non-blood group O, while individuals with blood group O were protected from contracting COVID-19 [19].

Several studies have been linked between blood group types and susceptibility and severity to bacterial,

parasitic and viral infection including severe acute respiratory syndrome (SARS-CoV-1) [22,30,31]. The susceptibility to SARS-CoV-1 infection (which is up to 70% similar to SARS-CoV-2 [32], has been linked to ABO polymorphism [22]. Other investigators have found the presence of anti-A antibodies could be a protective against SARS-CoV-1 [25,33]. This is mainly driven by anti-A antibody inhibiting the binding of angiotensin converting enzyme-2 to angiotensin converting enzyme-2—expressing on the cells [22].

Although, the exact mechanism of the interaction of ABO blood group and SARS-Cov2 is not fully understood and needs to be elucidated, several studies have postulated different mechanism for the influence of ABO blood groups on modulating COVID-19 infection and SARS-CoV-2 binding to the cells [34]. Silva-Filho et al. (2020) have linked the influence of ABO blood group on COVID-19 infection mainly blood group A through the modification of sialic acid containing receptors mainly modulation of the carbohydrate-carbohydrate interactions with host cells allowing potentially more binding of the virus (SARS-CoV-2) with host cell [34].

Other studies have suggested that receptor binding domain SARS-CoV-2, which is main part responsible for the COVID-19 infection [35] could bind to blood group A [36]. Moreover, a possible mechanism in reducing the susceptibility and severity of COVID-19 infection is attributed to the anti A and anti-B antibodies in non-blood group A, non-blood group B and non-blood group AB, which can inhibit the interaction between the spike protein of SARD-CoV-2 and the receptor on the target cells [25], or could be due to their ability to opsonize the virus [37]. Furthermore, the levels of anti-A and anti-B play a key role in the COVID-19 infection, as those will high levels of anti-A and anti-B antibodies lower COVID-19 infection [38–40]. COVID-19 infection could be also modulated by the host transmembrane protease serine subtype 2 [41,42].

The current study also showed elevated D-dimer levels in COVID-19 patients with prolonged PT and aPTT; consistent with the findings of previous studies [11,43]. Elevated D-dimer levels have been linked to severity and mortality in COVID-19 patients [44]. As D-dimers are produced through fibri nolysis by degrading the fibrin clots, hence its elevated levels indicates thrombotic tendency in COVID-19 patients [11]. Elevated D-dimer levels are suggested to be associated with disease compli cations, including COVID-19 severity. Elevated D-dimer levels have been proposed to be a surrogate and reliable prognostic marker of increased mortality among COVID-19 in hospitals and aiding in early intervention regimes among COVID-19 patients [45,46]. In the current study elevated D-dimer levels were more pronounced in blood group A as compared to non-blood group A.

The role of coagulation system in thrombotic tendency and manifestations of COVID-19 is not fully understood and needs to be elucidated. Furthermore, hypercoagulability is multifactorial, and RBC hemolysis is a known risk factor. RBC hemolysis contributes to hypercoagulability and the subsequent prothrombotic tendency in COVID-19. Our findings show the presence of anti-T antibody in patients with blood group A. Anti-T is a naturally occurring antibody against T-antigen on the RBC's membrane, renal endothelium and platelets [47]. In normal individuals with a normal physiological mechanism, the T-antigen has hidden receptors (crypt antigens) on the RBC's membrane. In several clinical conditions such as severe infections, malignancies, and due to some idiopathic reasons [19], the T-antigen on the RBC membrane is unmasked and exposed. RBC hemolysis externalizes the inner negatively charged membrane phospholipids to the outer surface, mainly phosphatidylserine (PS) [48]. RBCs with exposed PS have the ability to adhere to endothelial cells [49]. Furthermore, they enhance and support thrombin generation and tissue factor expression in many disorders including beta-thalas semia and sickle cell disease [50,51]. Thrombin is known to be a key player not only in haemostasis and thrombosis but also in driving inflammation, vascular endothelium regulation, and blood cells activation [52].

All of these conditions, we hypothesized, set a platform for thrombin to orchestrate fibrin for mation.

Once fibrin clot formation is initiated, the counterpart mechanism in human body; fibrinolysis, dissociates and breaks down the fibrin clot into fibrin degradation products (FDPs), where the D-dimer is part of these FDPs derived from cross linked fibrin.

The current study is a preliminary study that investigated the possible role of anti-T in driving the thrombotic tendency in COVID-19 and its link to ABO blood group types. However, similar to other studies, the study has some limitations to it, such as small sample size and lack of details related with co-morbidities.

5. Conclusions

Our data suggest that patients with blood group A are more susceptible to serious complications due to COVID-19, whereas patients with blood group O or B are less likely to suffer from severe complications due to COVID-19 as per their admissions in ICU. It is worthwhile to conduct this study on a large number of patients and look at affected markers and possible correlations with the ABO blood group type.

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Conflict of interest

All authors have no conflict of interest in this manuscript.

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The correlation between obesity and other cardiovascular disease risk factors among adult patients attending a specialist clinic in Kumasi. Ghana

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ABSTRACT

Background: *Obesity is a complex and multifactorial disease marker, which has become a major threat to cardiovascular health. We sought to assess the correlation of obesity and other cardio-metabolic risk factors in patients seen at the outpatient specialist clinic in Ghana. Methods: A prospective cross-sectional study was conducted on 395 patients at Precise Specialist Clinic in Kumasi, Ghana. A standardized questionnaire was used to obtain demographic, anthropometric and clinical data of patients. Fisher's exact test for statistical significance at a 95% confidence interval was used to evaluate associations between categorical variables. The associations between obesity indices and cardiovascular disease risk factors were analyzed by Pearson's correlation. Results: Of the 395 participants, 187 were males and 208 were females. The mean (± standard deviation) age of study participants was 59.29 (± 13.93); more than half of the participants were between 50 and 69 years. The mean BMI of male participants was significantly lower than the mean BMI of female participants (28.18 kg/m2 vs 31.16 kg/m2, P-value < 0.0001). Gender was significantly associated with the weight categories (P = 0.0144). Obesity was seen more in females (49.0%) than in males (35.8%). The Pearson correlation analysis also showed a significant positive correlation between obesity, increasing systolic blood pressure (r = 0.1568, P-value = 0.0018) and increasing diastolic blood pressure (r = 0.2570, P-value < 0.0001). Conclusions: Obesity was found to be significantly associated with female gender, increasing age, increasing systolic blood pressure, and increasing diastolic blood pressure. Efforts to step-up preventive measures to reduce the increasing prevalence of obesity in Ghana are highly recommended.*

Keywords: obesity; overweight; cardiovascular risk factors; blood pressure; dyslipidaemia cardiovascular diseases

1. Introduction

Overweight and obesity are complex and multifactorial disease marker, which has become a major threat to cardiovascular health in both economically endowed and less economically endowed countries of the modern world [1,2]. The World Health Organisation (WHO) defines overweight as a body mass index (BMI) ≥ 25 kg/m2; and obesity as a BMI ≥ 30 kg/m2 [3]. Raised BMI is a major risk factor for cardiovascular diseases (CVD) which remains the leading cause of death globally [4]. The increasing proportion of overweight and obesity in youth and adult life is likely to result in a high burden of obesity-mediated cardiovascular diseases worldwide.

Accordingly, the increasing emergence of overweight and obesity and its cardio-metabolic diseases may

be stemming from increasing urbanization and unhealthy lifestyles, which in turn has led to the emergence of a nutrition transition characterized by a shift to a higher calorie diet [5,6] This epidemic of obesity is paralleled by an alarming increase in the incidence of non-communicable comorbidities such as CVD and other chronic diseases such as type 2 diabetes mellitus, chronic kidney disease and many cancers [7–10]. Indeed, the risk factors that have been investigated and commonly recognized as also contributing significantly and independently to the global increase of overweight and obesity include hypertension, type 2 diabetes mellitus and dyslipidemia. However, several of the CVD risk factors are linked to each other, for example, physical inactivity contributes to overweight, which is a major risk factor for developing hypertension and type 2 diabetes mellitus.

In sub-Saharan African countries such as Ghana, the trend in overweight and obesity is increasing as excess weight is often considered to reflect healthy living, prestige, and affluence whilst the lean are perceived to be unhealthy or financially handicapped [4–6]. The work done by Ofori-Asenso et al is of particular interest for our knowledge of current trends in obesity-related disease incidence, and potentially also of what to expect in the future with increasing numbers of overweight and obesity among Ghanaian adults [11].

The increasing burden of overweight and obesity is a key risk factor and cardio-metabolic diseases in Ghana. Indeed, the high and rising burden of overweight and obesity should be relevant to nutritional scientists, health workers and the government of Ghana due to the impact on health and a high propensity of an explosion of chronic diseases such as metabolic syndrome, heart failure and stroke [3,11,12].

Numerous studies have shown a relationship between obesity and other cardiovascular disease risk factors. However, a regional-based evaluation of the relationship between obesity indices and cardio-metabolic risk factors is recommended, due to the regional variations observed by previous studies [13]. To provide current data and to support evidence-based policymaking, this study was done to determine the correlation between obesity and other common cardio-metabolic risk factors in Ghana.

2. Materials and methods

A prospective cross-sectional study was conducted at the Precise Specialist Clinic, which provides specialist medical and cardiac healthcare for adults in the Kumasi metropolitan of Ghana. Out-patients aged 18 years and above who presented at the clinic over a period of three (3) months were approached for voluntary participation.

We excluded children, patients on cancer treatment, and patients with congenital heart diseases. A Standardized questionnaire was used in obtaining the socio-demographic characteristics, disease history and physical examination findings of all the study participants. History of cardio-metabolic risk factors such as hypertension, type 2 diabetes mellitus, and dyslipidemia were also obtained. Body weight and height were measured with light clothes and bare feet using a combined weighing scale and stadiometer. Blood pressure was measured using the OMRON M6 devices with appropriate cuff sizes. Three blood pressure readings were taken from the left arm, with participants in the sitting position after a 10-minutes rest. The mean of the recorded readings was taken as the participant's blood pressure. Venepuncture was done from the antecubital veins in a recumbent position on all the participants, and 10mls of blood was collected into appropriate bottles for the determination of fasting blood glucose and lipid profile using an auto-analyzer at the biochemistry laboratory.

Hypertension was defined as the presence of a persistent elevated systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg in patients aged 15 years and above, and/or the presence of hypertensive retinopathy and/or the use of antihypertensive drugs and/or past medical history of hypertension [12]. Diabetes mellitus was defined as a random blood glucose level of 11.1 mmol/L or

greater, and/or fasting blood glucose level of 7.0 mmol/L or greater and/or use of insulin or an oral hypoglycemic agent [13]. Dyslipidemia was defined as low levels of high density lipoproteins (HDL) cholesterol (men ≤1.036 mmol/L, women ≤1.295 mmol/L) and/or high levels of low density lipoproteins (LDL) cholesterol ≥3.0 mmo/L and/or hypertriglyceridaemia ≥1.7 mmol/L [14]. Obesity/overweight was determined using the body mass index (BMI). The BMI was calculated as the weight of patients in kilograms divided by the square of the height in meters. Obesity and overweight were defined as a BMI ≥ 30 kg/m2, and a BMI ≥ 25 kg/m2 but <30 kg/m2 respectively [3].

2.1. Ethical consideration

Ethical approval (CHRPE/336/21) for the study was obtained from the Committee on Human Research, Publication and Ethics, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Informed consent was also obtained from all the study participants.

2.2. Statistical analysis

Data from the standardized questionnaire were entered into a Microsoft Excel (2016) sheet. Data were cleaned and abnormal variables and wrong entry removed or changed. Data were then exported into SPSS version 25.0 software and GrapPad Prism version 8.0 for statistical analysis. A descriptive analysis of baseline parameters was provided. Measure of central tendency using the mean was calculated, and measure of spread using standard deviation and range were also calculated. For all categorical valuables, bivariate analysis was done using Fisher's exact test for statistical significance. Pearson's correlation analyses were employed to determine the relationship between obesity indices and other cardiovascular risk factors. For all analyses P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Sociodemographic, anthropometric and cardiovascular risk factors of study participants stratified by gender

Table 1 shows the sociodemographic, anthropometric and cardiovascular risk factors of the study participants stratified by gender. Of the 395 participants, 187 were males and 208 were females. The mean (± standard deviation) age of study participants was 59.29 (± 13.93); more than half of the study participants were between 50 and 69 years. Three-quarters (75.5%) of the study population were either overweight or obese. The mean BMI of male participants was significantly lower than the mean BMI of female participants (28.18 kg/m2 vs 31.16 kg/m2, P < 0.0001). Gender was significantly associated with the weight categories (P = 0.0144). Obesity was seen more in females (49.0%) than in males (35.8%). The prevalence of type 2 diabetes mellitus (DM) was higher in the female population (26.9%) than the male population (17.6%), with a significant association between DM and the participant's gender (P = 0.0276). HDL-C level was significantly associated with the gender of participants (P = 0.0193), with the proportion of females having a low HDL-C level (23.6%) being higher than that of the males (12.3%).

Table 1. Sociodemographic, anthropometric and cardiovascular risk factors of study participants stratified by gender.

Variables	Total (n = 395)	Male (n = 187)	Female (n = 208)	Statistics	*P*-value
Mean age (Years)	59.29 ± 13.93	57.98 ± 13.81	60.6 ± 14.09		0.0619
24–39	40 (10.1)	21 (52.5)	19 (47.5)		
40–49	55 (13.9)	29 (52.7)	26 (47.3)		
50–59	101 (25.6)	52 (51.5)	49 (48.5)		
60–69	107 (27.1)	48 (44.9)	59 (55.1)		
70–79	71 (18.0)	29 (40.8)	42 (59.2)		
80–89	21 (5.3)	8 (38.1)	13 (61.9)		
Mean SBP (mmHg)	130.1 ± 19.36	131.3 ± 19.21	129.2 ± 20.71	t = 0.7534	0.4519
Mean DBP (mmHg)	80.0 ± 12.41	80.1 ± 12.32	79.96 ± 12.61	t = 0.0819	0.9348
Mean BMI (kg/m^2)	29.67 ± 5.84	28.18 ± 5.289	31.16 ± 7.618	t = 4.459	**<0.0001**
BMI status				8.483, 2	**0.0144**
Normal weight	97 (24.6)	56 (57.7)	41 (42.3)		
Overweight	129 (32.7)	64 (49.6)	65 (50.4)		
Obese	169 (42.8)	67 (39.6)	102 (60.3)		
TG	1.15 ± 0.45	1.15 ± 0.44	1.15 ± 0.52	t = 0.0290	0.9769
HDL-C	1.35 ± 0.46	1.29 ± 0.42	1.41 ± 0.51	t = 1.848	0.0659
LDL-C	2.96 ± 1.05	2.84 ± 1.09	3.07 ± 1.26	t = 1.395	0.1645
TC	4.72 ± 1.21	4.63 ± 1.24	4.80 ± 1.20	t = 0.6946	0.4885
TG levels				0.2516, 1	0.6160
High	33 (8.4)	17 (51.5)	16 (48.5)		
Desirable	362 (91.6)	170 (47.0)	192 (53.0)		
HDL-C level				5.474, 1	**0.0193**
Low	72 (18.2)	23 (31.9)	49 (68.1)		
Desirable	349 (88.4)	164 (47.0)	185 (53.0)		
Dyslipidaemia				0.1515, 1	0.6971
Yes	47 (11.9)	21 (44.7)	26 (55.3)		
No	348 (88.1)	166 (47.7)	182 (52.3)		
HTN				3.530, 1	0.0603
Yes	241 (61.0)	105 (43.5)	136 (56.4)		
No	154 (39.0)	82 (53.2)	72 (46.8)		
DM				4.854, 1	**0.0276**
Yes	89 (22.5)	33 (37.1)	56 (62.9)		
No	306 (77.5)	154 (50.3)	152 (49.7)		

Note: P-value: Probability value; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; TG: Triglycerides: HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; TC: Total cholesterol; HTN: Hypertension; DM: Type 2 diabetes mellitus.

3.2. Prevalence of hypertension and type 2 diabetes mellitus in participants with normal weight, overweight and obesity

Table 2 shows the prevalence of hypertension and DM in participants with normal weight, overweight and obesity. Hypertension was significantly associated with the weight categories (P = 0.0001) with 74.6% of obese patients and 53.5% of overweight patients having a history of hypertension. DM was not significantly associated with the weight categories with only 20.9% of DM patients being overweight and 25.4% of DM patients being obese. Systolic blood pressure (SBP) increased with an increasing BMI (P = 0.0443), with obese individuals having the highest SBP (Table 2). Diastolic blood pressure (DBP) also shows a significant elevation as the BMI increased (P < 0.0001).

Table 2. Prevalence of hypertension and diabetes in healthy weight, overweight and obese categories.

Variables	Normal (n = 97)	Overweight (n = 129)	Obese (n = 169)	Statistics	*P-value*
Mean age	61.66 ± 16.32	58.16 ± 13.42	58.81 ± 12.93	*F*, 1.91	0.1494
Age range n (%)				χ22.40,10	**0.0132**
34–39	14 (14.4)	13 (10.1)	13 (7.7)		
40–49	6 (6.2)	19 (14.7)	30 (17.8)		
50–59	19 (19.6)	38 (29.5)	42 (24.9)		
60–69	23 (23.7)	33 (25.6)	50 (29.6)		
70–79	23 (23.7)	19 (14.7)	28 (16.6)		
80 and above	12 (12.4)	7 (5.4)	6 (3.6)		
Gender n (%)				χ8.483, 2	**0.0144**
Male	56 (57.7)	64 (49.6)	67 (39.6)		
Female	41 (42.3)	65 (50.4)	102 (60.4)		
SBP	125.3 ± 22.48	129.7 ± 20.64	132 ± 20.64	*F*, 3.141	**0.0443**
DBP	75.19 ± 12.37	81.09 ± 12.80	82.51 ± 11.32	*F*, 11.78	**<0.0001**
History of HTN n (%)				χ17.92, 2	**0.0001**
Yes	49 (50.5)	69 (53.5)	126 (74.6)		
No	48 (49.5)	60 (46.5)	43 (25.4)		
History of DM n (%)				χ1.487, 2	0.4753
Yes	18 (18.6)	27 (20.9)	43 (25.4)		
No	79 (81.4)	102 (79.1)	126 (74.6)		

Note: P-value: Probability value; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HTN: Hypertension; DM: Type 2 diabetes mellitus.

3.3. Prevalence of dyslipidaemia in normal weight, overweight and obesity

Table 3 shows the lipid profile characteristics of participants. Mean triglyceride (TG) level, mean HDL-cholesterol (HDL-C), mean LDL-cholesterol (LDL-C) and mean total cholesterol (TC) were not significantly different among the 3 weight categories (P > 0.05). Obese patients had higher proportions of high TG levels (10.7%) and low HDL status (21.9%) as compared to overweight patients (9.3% and 20.2%) and normal weight patients (3.1% and 8.2%) respectively. Both TG level and HDL status were however not associated with patient weight category P > 0.05). Dyslipidaemia s was significantly associated with weight category (P = 0.0104) with higher proportions (30.2%) among the obese patients

(26.4%) and patients with normal weight (1.0%).

Table 3. Lipid profile Characteristics among outpatients.

Variables	Normal (n = 97)	Overweight (n = 129)	Obese (n = 169)	Statistics	*P*-value
TG (mmol/l)	1.04 ± 0.37	1.20 ± 0.56	1.16 ± 0.46	*F*, 1.326	0.2677
HDL-C (mmol/l)	1.42 ± 0.40	1.32 ± 0.45	1.36 ± 0.50	*F*, 0.5647	0.5647
LDL-C (mmol/l)	3.04 ± 1.19	2.84 ± 1.22	2.99 ± 1.17	*F*, 0.4892	0.4892
TC (mmol/l)	4.92 ± 1.19	4.65 ± 1.37	4.86 ± 1.35	*F*, 0.7191	0.4884
TG levels n (%)				χ2.061, 2	0.3568
High	3 (3.1)	12 (9.3)	18 (10.7)		
Desirable	94 (96.9)	117 (90.7)	151 (89.3)		
HDL-C status n (%)				χ3.213, 2	0.2006
Low	8 (8.2)	26 (20.2)	37 (21.9)		
Desirable	89 (91.8)	103 (79.8)	132 (78.1)		
Dyslipidaemia n (%)				χ9.125, 2	**0.0104**
Present	1 (1.0)	34 (26.4)	51 (30.2)		
Absent	96 (99.0)	95 (73.6)	118 (69.8)		

Note: P-value: Probability value; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; TC: Total cholesterol.

3.4. Pearson correlation of BMI with lipid markers and blood pressure characteristics among the study participants

Table 4 shows the Pearson correlation of BMI with lipid markers and blood pressure characteristics among the study participants. BMI showed a significant positive correlation with systolic blood pressure (r = 0.1568, P = 0.0018), diastolic blood pressure (r = 0.2570, P < 0.0001). The correlation of BMI with total cholesterol, triglycerides, high density lipoprotein cholesterol and low density lipoprotein cholesterol was however not significant.

Table 4. Pearson correlation between BMI and lipid markers and blood pressure characteristics among study participants

Pearson correlation	R	95% confidence interval	R squared	*P*-value
BMI vs. SBP	0.1568	0.0591 to 0.2516	0.0246	**0.0018**
BMI vs. DBP	0.2570	0.1624 to 0.3468	0.0660	**<0.0001**
BMI vs. TC	−0.0109	−0.1445 to 0.1231	0.0001	0.8736
BMI vs.TG	0.0364	−0.0985 to 0.1700	0.0013	0.5969
BMI vs. HDL-C	−0.0285	−0.1620 to 0.1060	0.0008	0.6787
BMI vs. LDL-C	−0.0085	−0.1427 to 0.1261	7.14E-05	0.9024

Note: P-value: Probability value; R: Pearson correlation coefficient; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TC: Total cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.

4. Discussion

This study provided a detailed finding of the correlation between obesity and other cardiometabolic risk factors in an outpatient specialist clinic. Overweight or obesity is a strong risk factor for CVD and tends to be associated with other cardio-metabolic risk factors [15–17]. In this study, associations were identified between obesity and increasing age, female gender, increasing SBP and DBP. These findings are essential in aiding healthcare professionals and policymakers in healthcare planning and for improving healthcare services in Ghana and other sub-Saharan African countries.

A total of 395 patients (male 48% and female 52%) were recruited for the study. The findings of this study show that three-quarters (75.5%) of the adults who visited the clinic during the study period had high BMI and about 43% were classified as obese by the WHO definition [3]. The very high prevalence rate of overweight and obesity observed in this study compares well with the findings of similar studies by Cil et al. [18] and Kossaify et al. [19] who recorded 76% and 72% in their respective study populations. A recent systematic review reported that about 43% of the Ghanaian population is either overweight or obese [11], which is comparable to the prevalence rate worldwide.

Consistent with the findings of previous studies in other countries [20–22], this study found that the prevalence of obesity increased with age but started to decline at 70 years in women and at 60 years in men. Studies by Yusuf et al. [23] showed that after middle age, body fat accumulation began to increase with age and tended to accumulate in certain areas of the body. Although the pattern of body fat accumulation is similar in different countries, there are still some differences. Body fat percentages differ among countries depending on genetic factors, eating patterns, regular exercise, and other lifestyle habits [24]. Previous research work reported that weight gain in adulthood appears to increase the risk for colon cancer [25]. Valdes et al. [26] pointed out that obesity is not only related to shortened life expectancy, but also it accelerates aging. Therefore the promotion of healthy lifestyles in order to prevent weight gain is very essential.

It has been shown by previous studies that the gender effect on obesity varies from population to population, irrespective of the anthropometric measurement used for its assessment [27–29]. This study found that the female gender was strongly associated with obesity. This pattern is consistent with findings from a previous series by Ofori-Asenso et al. [11] and Abubakari et al. [30] in which the female gender was strongly related to obesity than their male counterparts. The higher prevalence of obesity among females than males shown in this study is also consistent with globally observed gender differences among obesity subjects [3].

Even though the reason for this gender difference is not immediately obvious, these differences might be due to the fact that females generally tend to gain weight as a result of the hormonal effect on the redistribution of body fat [27,28]. Furthermore, studies have documented that many Ghanaian communities show great admiration towards obese individuals [31,32]. Often overweight or large body size is regarded as a sign of “affluence” and women also tend to perceive this as signifying “good nutrition, healthy life, beauty and marital happiness” [33]. Needless to mention, among Ghanaian women, “plumpness” tends to be the culturally preferred body size seen as a symbol of well-being. Further epidemiological studies will be needed to fully elucidate the sex differences in the prevalence of obesity.

Overweight and obesity are major risk factors for the development of DM, and many epidemiological studies have suggested a progressive increase in the prevalence of DM with obesity [34]. Even though DM was not significantly associated with overweight and obesity, it was seen in 20.9% and 25.4% of overweight and obese individuals in this study. The absence of a correlation between overweight/obesity and DM in our study could be due to the sample size not being large enough to be empowered to detect a significant association.

Hypertension prevalence rates of 74.6% and 53.5% were seen among obese and overweight individuals respectively. A strong positive association between obesity and increasing SBP and DBP was found in this study using Pearson's correlation analysis. A variety of population studies have clearly established that obesity is strongly correlated with high blood pressure [16,17,35,36]. Poston et al. found that obesity was consistently related to high blood pressure, but not other CVD risk factors, in a cohort of 478 Missouri Valley firefighters [36]. Hypertension was demonstrated to be strongly associated with obesity in a study involving South African adolescents aged 13–17 years [37]. There is a strong established link between obesity and hypertension [16,38–41]; and hypertension has been found to be the main driver of CVD morbidity and mortality [41–43]. Studies have shown that obese individuals who are aged 65 years and above are more likely to be hypertensive compared with individuals aged 40 years or less [32].

The growing prevalence of obesity is increasingly recognized as one of the most important risk factors for the development of hypertension. Currently, hypertension is driving the high burden of CVD, and this is partly due to the increasing burden of the global obesity prevalence rate [3]. Several mechanisms have been postulated as potential explanations for the mechanisms contributing to the development of higher blood pressure in obese individuals. These include activation of the renin–angiotensin–aldosterone system, hyperinsulinemia, sympathetic stimulation, leptin resistance. stimulation of procoagulatory activity and endothelial dysfunction [44–47]. However, the exact mechanisms of the relationship between obesity and hypertension are still not fully understood.

Dyslipidemia is one of the most important causal risk factors for atherosclerotic vascular disease. Our study however did not show a positive association between obesity and dyslipidemia. In contrast with this study, epidemiologic studies establishing and describing the relationship between obesity and dyslipidemia are extensive and well documented [39,40,48]. Several studies have demonstrated the evidence of dyslipidemia as an important CVD risk factor; a major cause of atherosclerosis and adverse cardiovascular events [49,50]. Dyslipidemia acts synergistically with other risk factors, substantially increasing the risk of cardiovascular events. There is substantial evidence demonstrating that the trajectory of atherosclerotic vascular disease can be greatly improved by lowering blood lipid levels [50,51].

The authors acknowledge some limitations in this study. First, some potential confounding factors that were not obtained in our study might have affected the study's findings. Secondly, the study design was cross-sectional, and thus cannot determine causal relationships, though the sample was quite diverse and large enough.

Overall, the high and rising prevalence of obesity should be a major wake-up call for stakeholders including nutritional scientists and healthcare workers due to the impact on health and a possibility of an explosion of Cardio-metabolic risk factors and disease.

5. Conclusions

This study found a significant correlation between obesity and increasing age, female gender, increasing SBP, and increasing DBP, which are compendiums of key risk factors for cardiovascular events. Strategies including health promotion programs and lifestyle changes such as healthy eating and increased physical activity are highly recommended.

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Authors' contributions

All authors made a significant contribution to this study, whether that is in conception, study design, execution, data collection, data analysis and interpretation. All authors also took part in the drafting, revising, and gave approval for the publication of this manuscript.

Conflict of interest

The authors confirm that there are no conflicts of interest in this article's content.

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Increased risk of diabetic ketoacidosis in an Urban, United States, safety-net emergency department in the COVID-19 era

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ABSTRACT

Aims: *The incidence of diabetic ketoacidosis (DKA) increased during the COVID-19 pandemic but estimates from low-resource settings are limited. We examined the odds of DKA among emergency department (ED) visits in the Los Angeles County Department of Health Services (DHS) (1) during the COVID-19 pandemic compared to the pre-COVID era, (2) without active COVID infections, and (3) stratified by effect modifiers to identify impacted sub-groups. Methods: We estimated the odds of DKA from 400,187 ED visits pre-COVID era (March 2019–Feb 2020) and 320,920 ED visits during the COVID era (March 2020–Feb 2021). Our base model estimated the odds of DKA based on the COVID era. Additional specifications stratified by effect modifiers, controlled for confounders, and limited to visits without confirmed COVID-19 disease. Results: After adjusting for triage acuity and interaction terms for upper respiratory infections and payor, the odds of DKA during the COVID era were 27% higher compared to the pre-COVID era (95%CI 14–41%, $p < 0.001$). In stratified analyses, visits with private payors had a 112% increased odds and visits with Medicaid had a 20% increased odds of DKA during the COVID era (95%CI 7–36%, $p = 0.003$). Conclusions:We identified increased odds of DKA during the COVID pandemic, robust to a variety of specifications. We found differential effects by the payor; with increased odds during COVID for privately-insured patients.*

Keywords: DKA; emergency department; COVID-19; pandemic; insurance; access to care

1. Introduction

Diabetic ketoacidosis (DKA) is an endocrine emergency, commonly triggered by infection, cardiovascular events, catecholamine surges such as with extreme stress, or discontinuation of insulin administration. During the COVID-19 pandemic, the incidence of DKA has increased [1–3]. This increase is postulated to be due to several reasons: increased rates of new diagnosis of type I diabetes after acute COVID-19 infection, inflammatory responses to COVID in patients with existing diabetes, and with secondary factors related to the pandemic such as inadequate access to care or acute stress [4–6]. However, our understanding of the relationship of these factors is limited in low-resource and adult populations, which might be more susceptible to ambulatory care-sensitive conditions, such as DKA and hyperglycemia. Understanding their relative importance will allow for better planning for future pandemics as well as preparing for the transition of this pandemic to the endemic phase.

To better understand the causes of the increased incidence of DKA in an urban safety-net population living in the US, we examined trends in DKA at a large public health system in the context of the COVID-19 pandemic. We first examined if the odds of a diagnosis of DKA increased among adult emergency department (ED) patient visits in the COVID era compared to the pre-COVID era, after adjusting for relevant confounders. We then examined if these increased odds of DKA were sensitive to the removal of visits from patients with active COVID infections. Lastly, we evaluated the odds of DKA stratified by significant effect modifiers to identify sub-groups most impacted by the phenomena.

2. Methods

2.1. Study design and setting

This is a retrospective analysis of all ED encounters in the Los Angeles County Department of Health Services (DHS) for the one-year period preceding the arrival of the community spread of COVID-19 in Los Angeles County (March 2019 to February 2020) and the first one-year period of the COVID-19 pandemic and associated social distancing measures in Los Angeles County (March 2020 to February 2021). COVID vaccinations were not widely available until after the study period ended. Adult patients 18 years or older at the time of the ED visit were included. Outcomes and patient visit characteristics were collected from the electronic health records (EHR) of patients during these visits. The Los Angeles County DHS system contains 3 EDs located in separate medical centers.

2.2. Measures

The primary outcome of this study was the categorization of an ED visit as having been caused by DKA as identified by an ED ICD-10 diagnosis in the EHR. We categorized an ED encounter as a DKA case if the first five listed diagnoses included Diabetic Ketoacidosis (ICD10 codes E10.10, E11.10, E11.11, E13.10, or E13.11). The primary independent variable of interest was the time period of exposure. The "pre-COVID" period was defined as March 2019 to February 2020, while the "COVID"-era period was defined as March 2020 to February 2021.

As described in prior work with this dataset [7], patient reported demographic characteristics were collected from patients during ED registration, including patient age; gender; race or ethnicity, primary language and Emergency Severity Index (ESI) triage category. The presence of a respiratory illness was defined by ICD-10 diagnosis, and our methodology for this categorization has been described previously [7]. These patient and encounter-level characteristics are presented as the count or proportion of all weekly ED visits accounted for by these categories. We defined a COVID infection as a positive PCR or antigen test from medical center laboratory records. All patients with DKA were tested for COVID infection prior to admission. Patients were not retested while hospitalized per hospital policy.

2.3. Statistical analysis

Patient and visit characteristics were described in tables and graphically displayed using histograms. All administrative variables had less than 2% missingness and were included with a plan for listwise deletion for multivariate models. ESI/triage acuity scale was categorized as 1–2 (indicating high acuity), 3 and 4–5 (indicating lower acuity) given the low numbers in triage category 1 and 5. The primary language was categorized as English, Spanish and "Other" give the low numbers of individual languages other than English or Spanish. Insurance was categorized as Medicaid, Medicare, Private, None and Other Government according to algorithms used in our previous work [7].

We examined the patient and patient visit characteristics across and between the study period with descriptive analysis (Table 1). We then used logistic regression models to estimate the odds of DKA from the pre-COVID period (March 2019–Feb 2020) vs the COVID-era period (March 2020–Feb

2021). We started with a base model of the probability of a patient visit being diagnosed as DKA modeled on time period alone (Figure 1, Bar 1). We then checked for potential confounding by modifying the base model with each individual potential confounder. Terms that changed the regression coefficient for time period by a substantial amount (greater than 15%) from the base model were retained in the final model. We examined insurance payor, patient age, ESI triage acuity, gender, presence of respiratory infection, patient reported race and ethnicity and patient primary language as potential confounders. We also examined these candidate variables as potential effect modifiers with interaction terms, retaining them if the Wald test for the interaction term regression coefficient was statistically significant. We calculated odds ratios stratified by subgroups for significant effect modifiers. Lastly, we conducted a sub-analysis to examine the indirect effects of the COVID pandemic on the odds of DKA—such as reduced access to ambulatory care—by removing patients who had a confirmed diagnosis of COVID19. We used a p-value of <0.05 to indicate statistical significance. For multiple comparisons in the subgroup analysis of significant effect modifiers, we used the Bonferroni correction.

2.4. Ethics approval of research

This work was approved by the University of Southern California Institutional Review Board, approval UP-20-00344-AM002.

3. Results

Among 720,477 Adult ED visits identified in the EHR in the study period, there were 1,395 cases of DKA (Table 1), with a similar number of cases of recurrent DKA. The pre-COVID era population was of similar age to the COVID era population and was slightly more likely to be male. The COVID era population had the same rate of Medicaid insurance, higher rates of Medicare and private insurance and lower rates of no insurance and other government program insurance than the pre-COVID population. The COVID era population had higher proportions of triage acuity (ESI) scores in the ranges of 1–2 indicating higher acuity, and a higher proportion of visits attributable to upper respiratory infections (Table 1). The odds of a patient visit having a diagnosis of DKA were 40% higher in the COVID era than in the pre-COVID era (95% CI 1.26–1.55, $p < 0.001$; Figure 1, Bar 1).

Table 1. Visit and study population characteristics.

Visit characteristics	Pre-COVID-era Mar 2019–Feb 2020	COVID-era Mar 2020–Feb 2021	Combined eras
Total number of Visits	400,187	320,290	720,477
Visits with DKA diagnosis	658	737	1,395
Unique patients with DKA	368	367	689***
Visits for recurrent DKA	452	447	899
Visits with COVID diagnosis	0	10,624	10,624
Age (mean, sd)	46.94 (16.46)	46.60 (16.36)	46.78 (16.42)
Female Gender, % (n)	46.4% (185,547)	44.1% (141,076)	45.3% (326,623)
Race and Ethnicity, % (n)			
Hispanic/Latino	66.3% (265,176)	66.2% (212,058)	66.2% (477,234)
Black	12.6% (50,447)	12.8% (41,135)	12.7% (91,582)
Other and multi racial	11.4% (45,602)	11.5% (36,971)	11.5% (82,573)
Asian/Pacific Islander	4.9% (19,514)	4.4% (13,953)	4.6% (33,467)
Non-Hispanic White	4.9% (19,448)	5.1% (16,173)	4.9% (35,621)
Language, % (n)			
English	52.4% (209,645)	53.1% (169,920)	52.7% (379,565)

Spanish	43.5% (174,009)	42.9% (137,432)	43.2% (311,441)
Other	2.7% (10,857)	2.6% (8,426)	2.7% (19,283)
Missing	1.4% (5,676)	1.4% (4,512)	1.4% (10,188)
Insurance, % (n)			
Medicaid	67.3% (269,238)	67.3% (215,541)	67.3% (478,241)
Medicare	9.0% (36,034)	10.0% (32,085)	9.5% (67,161)
Private	5.6% (22,575)	6.4% (20,619)	6.0% (34,480)
Other	10.9% (43,737)	9.3% (29,753)	10.2% (72,651)
None	6.4% (25,461)	5.9% (18,847)	6.2% (31,248)
Missing	0.8% (3,142)	1.1% (3,445)	0.9% (6,587)
Triage Acuity Score, % (n)			
1–2	19.0% (76,182)	23.2% (74,421)	20.9% (150,603)
3	57.1% (228,620)	56.0% (179,243)	56.6% (407,863)
4–5	22.2% (88,872)	19.1% (61,142)	20.8% (150,014)
Missing	1.6% (6,513)	1.7% (5,484)	1.7% (11,997)
Presence of Upper Respiratory Infection, % (n)	6.7% (26,761)	9.8% (31,268)	8.1% (58,029)

In our multivariate analysis, 17,704 visits (2.5% of visits) were excluded for missing one or more data points. We found the triage acuity score and the presence of respiratory infection diagnosis to substantially confound the association between the study period and DKA diagnosis, decreasing the regression coefficient by 29% and 42% respectively. Additionally, we found medical insurance type and the presence of respiratory infection diagnosis to be significant effect modifiers of the relationship between the study period and DKA diagnosis. Our final model included terms for triage acuity score and presence of respiratory infection as confounders and interaction terms for insurance type and presence of respiratory infection diagnosis as effect modifiers. After adjusting for triage acuity score and interaction terms for the presence of upper respiratory infections and type of insurance, the adjusted odds of DKA in the COVID period were attenuated to a 27% increased odds of DKA compared to the pre-COVID era (95%CI 14–41%, $p < 0.001$; Figure 1, bar 2).

In stratified analysis by insurance type, (Figure 1, bars 4–8) we found that visits with private insurance had a 112% increased odds of DKA in the COVID-era compared to the pre-COVID era (95%CI 40–220% increased odds, $p < 0.001$, pBonferroni ≤ 0.001). Visits with Medicaid insurance had a 20% increased odds of DKA in the COVID era (95%CI 7–36%, $p = 0.003$, pBonferroni = 0.015). Visits with other insurance had a 60% increased odds of DKA in the COVID era, however, this was not significant after adjusting for multiple comparisons (95%CI 6–142%, $p = 0.027$, pBonferroni = 0.054). There were no significant changes in the odds of DKA between the study periods for visits with Medicare or no insurance.

We examined the role of respiratory infection on odds of DKA by stratified analysis by respiratory infection followed by a sensitivity analysis excluding COVID cases. In stratified analysis by the presence of respiratory infection (Figure 1, bars 8&9), visits with respiratory infection had a 92% (95%CI 33–177%) increased odds of DKA in the COVID era, while visits without respiratory infection had a 21% (10–36%) increased odds of DKA in the COVID period. In our sensitivity analysis removing the 10,499 visits with COVID diagnosis from the COVID period, using the final multivariate regression model, we found a persistent 13% increased odds of DKA in the COVID period (95%CI 0.2–30%, $p = 0.045$; Figure 1, bar 3).

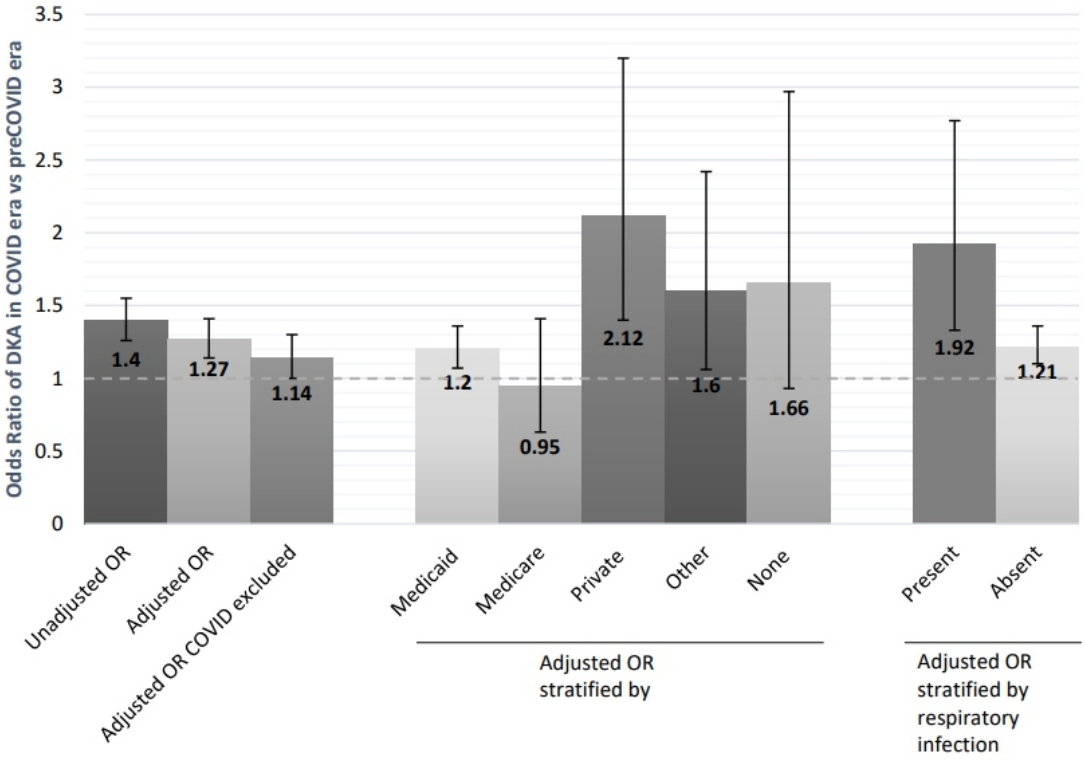

Figure 1. Unadjusted, Adjusted and Stratified Odds Ratios of DKA in COVID era vs PreCOVID Era.

4. Discussion and conclusions

In this novel analysis conducted in a low-resource population, we found increased odds of DKA in the COVID era compared to the pre-COVID era, robust to controlling for confounders and subgroup analysis removing visits with COVID infection. Additionally, we found different odds ratios for different medical insurance types; increased odds of DKA in the COVID era were significantly higher for private medical insurance.

The observed increased odds of DKA were robust to controlling for relevant confounders, and also to a sensitivity analysis removing COVID-era patient visits with a diagnosis of COVID. These findings suggest that it is not the pro-inflammatory response to COVID infection alone that caused increased DKA, but also non-physiologic factors such as reduced access to care, or patients delaying care due to fear of obtaining a COVID infection in a healthcare setting, factors seen in varying degrees in other populations [8–11]. The increased odds of DKA were most pronounced among patients with private insurance, suggesting that differential access to care associated with clinic closures and patients delaying care was greatest for those with private insurance, leading to increased DKA rates, while those with less access to care prior to the pandemic had less change with clinic closures.

While access to care may have a role in the increase in DKA found in our population, direct infection by COVID likely played a role as well. Effect modification by the presence of a respiratory infection (including COVID infection) does indicate there is a large role of the pathologic inflammation seen in patients with active COVID infections, as has been seen in other

populations [2,8,12,13]. Additionally, there is published data to support likely a role of new onset diabetes associated with direct beta cell destruction as well as an autoimmune response to COVID infection [12,14–18].

While presenting important findings on access to care in an underrepresented population, there are several limitations to our study, primarily related to observational nature of the study and the EHR/administrative dataset. The data source of ED-based EHR records results in underreporting of chronic conditions, and we do not know if patient visits for DKA represent a new diagnosis of diabetes or exacerbations of existing diagnoses. Additionally, chronic conditions and comorbidities that might predispose to DKA were not systematically captured in the EHR. This missing information includes prior insulin use and type of diabetes, limiting our ability to examine the phenomena of DKA in people with previously non-insulin dependent diabetes. Prospective studies in this population would improve this understanding, especially of the time-lag between COVID infection and development of DKA. This dataset was limited to the year immediately preceding the first COVID year, and we could not assess for previous trends in DKA. Lastly, our findings cannot be interpreted as causal relationships, but should inspire future work to better understand the rising rates of DKA in the COVID era.

Our findings on the role of access to care in the increased rates of DKA are important for health system leaders as the United States heads towards and endemic phase of COVID. The US has greatly increased telehealth capacity, especially for chronic diseases, and these changes must be sustained. However, increased rates of DKA directly related to COVID infection must also be considered, given the high level of nursing care required by DKA patients, particularly with current staffing shortages and prolonged boarding in the ED by ICU patients. Additional nursing resources and training are potentially necessary maybe necessary to plan for potential COVID surges in the coming year as we move to an endemic pattern of COVID-19 infection.

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Conflict of interest

The authors confirm that there are no conflicts of interest in this article's content

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Thyrotoxic periodic paralysis together with thyrotoxic heart disease in a Ghanaian man: case report and literature review

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ABSTRACT

Thyrotoxic periodic paralysis (TPP) is an uncommon symmetrical paralysis usually affecting proximal muscles, which occurs in the hyperthyroid state with associated hypokalemia. It is more prevalent in East Asian males and extremely rare in blacks. Data on TPP is scarce in Africa and no report has been made in Ghana. We report a case of a middle-aged Ghanaian man who had three episodes of paralysis in all four limbs occurring at night with the second and third episodes requiring hospital visit. He had no clinical signs of hyperthyroidism during his first hospital visit but had developed clinical and biochemical evidence of hyperthyroidism on the second visit with serum potassium levels of 1.9 mmol/l; and he was eventually diagnosed with TPP. His paralysis resolved with correction of the hypokalemia. It is important to evaluate patients presenting with paralysis comprehensively. Less common differential diagnosis such as TPP may also be considered in such patients to ensure early diagnosis and treatment which can prevent complications.

Keywords: thyrotoxic; periodic; paralysis; hypokalemia; case report

1. Introduction

Thyroid hormones are required by all nucleated cells in the body for normal metabolism. However, excess blood thyroid hormones leading to thyrotoxicosis causes dysfunction of various systems in the body including the cardiovascular, nervous, musculoskeletal and other systems [1]. Thyrotoxicosis has a prevalence of 0.2–1.3% in developed countries where iodine is supplemented [2]. In Africa data on population-based true prevalence of thyrotoxicosis is lacking. However, in Ghana, thyroid disorders account for 13% of endocrine clinic visits [3].

One rare neurological complication of thyrotoxicosis is thyrotoxic perioid paralysis (TPP) [4]. TPP is a sporadic disease characterized by hypokalemia with acute reversible recurrent flaccid muscle weakness in patients with thyrotoxicosis due to increased shift of potassium into cells with the weakness usually lasting a few hours to three days [5]. TPP commonly affects Asian males having an incidence of 1.8 to 1.9% in this population [6,7] and an estimated incidence of 0.1% to 0.2% in Caucasians [5]. Increasingly, cases are being reported outside Asia attributable to increased migration and the fact that clinicians are increasingly becoming aware of the condition [8].

TPP presents similarly to other forms of hypokalemic periodic paralysis (HPP) such as familial hypokalemic periodic paralysis (FPP) and other acquired forms of HPP but differentiated on the basis of biochemical evidence of hyperthyroidism [9].

Treatment involves correcting the hypokalemia with intravenous or oral potassium, giving a non selective beta blocker to stabilize the sodium-potassium ATPase and in the long term, treating the underlying thyrotoxicosis [10].

Although TTP is generally rare in blacks; a few cases have been reported in people of Afro origin living outside Africa [8,11–13] and also Africans living in Africa including Tanzania [14], Senegal [15] and Somalia [16]. However, no such report has been made in Ghana. We report a case of an adult Ghanaian black man who was eventually diagnosed with TPP after a year of delayed diagnosis.

2. Case presentation

A 43-year-old Ghanaian man presented to the Emergency Department of Komfo Anokye Teaching Hospital in Kumasi, Ghana at dawn with a third episode of weakness in all limbs which he noticed after waking up from sleep that dawn. He first noticed the weakness in his lower limbs which later progressed to involve his upper limbs a few hours prior to presentation.

He reported experiencing two similar episodes also occurring at dawn a year earlier. The first episode resolved spontaneously at home within a few hours. The second episode persisted for hours for which he was admitted at the hospital but regained full power spontaneously within a few hours on admission, discharged and followed up on out-patient basis but was lost to follow up.

He had not had any more paralysis over a year period until this third episode of paralysis that led to his second hospital admission. He had a preceding two months history of palpitations, dyspnea on minimal exertion and significant weight loss. He however was not on any medications, had no preceding diarrhea or vomiting and had no known history of any chronic illness. He had not taken an unusual amount of carbohydrate diet the preceding evening.

On examination, he was anxious, afebrile, anicteric and not pale. He had mild proptosis with thyroid eye disease clinical activity score of 1/10, diffuse goiter, tremulous hands and pitting bipedal edema. He was tachycardic with irregular pulse of 154 beats per minute (bpm) and a pulse deficit of 30 bpm. His blood pressure was 154/74 mmHg with respiratory rate of 22 cycles per minute. He had thrusting displaced apex beat, ejection systolic mummer and bibasal fine crackles. He was fully conscious, cranial nerves intact, power of 3/5 in both lower limbs and 4/5 in the upper limbs, with reduced muscle tone and deep tendon reflexes. The results of his investigations summarized in Table 1, Figures 1 and 2 below showed Graves' disease with severe hypokalemia (serum potassium of 1.9 mmol/l) and also dilated cardiomyopathy with reduced left ventricular systolic function and atrial fibrillation.

Table 1. Review of laboratory and imaging data.

Serum creatinine (umol/l)	81	-	75	44–106
Serum urea (mmol/l)	4	-	5	2.1–8.3
Serum potassium (mmol/l)	1.9	4.4	4.2	3.5–5.5
Serum sodium (mmol/l)	137	140	141	135–145
Serum chloride (mmol)	80	90	98	95–110
TSH (uIU/ml)	<0.015	<0.015	0.9	0.5–5.0
Free triiodothyronine (pmol/l)	>35	16	9	4.6–9.7
Free tetraiodothyronine (pmol/l)	>90	23	16	12–23
Anti TSH-receptor antibody (IU/l)	310.5	-	-	1.2–1.5
Thyroid ultrasonography	Diffusely enlarged thyroid gland with increased blood flow on color Doppler.			
Electrocardiogram	Atrial fibrillation with rapid ventricular response, left ventricular hypertrophy, ST/T changes and poor R wave progression			
Echocardiogram	Dilated cardiac chambers, global hypokinesia with LVEF of 45%, right ventricular systolic dysfunction (TAPSE 10 mm), normal valvular morphology with moderate to severe mitral and tricuspid regurgitation and severe pulmonary hypertension (RVSP 66 mmHg).			

Note: TSH: Thyroid stimulating hormone; LVEF: Left ventricular ejection fraction; TAPSE: Tricuspid annular plane systolic excursion; RVSP: Right ventricular systolic pressure; ST/T: ST segment and T wave.

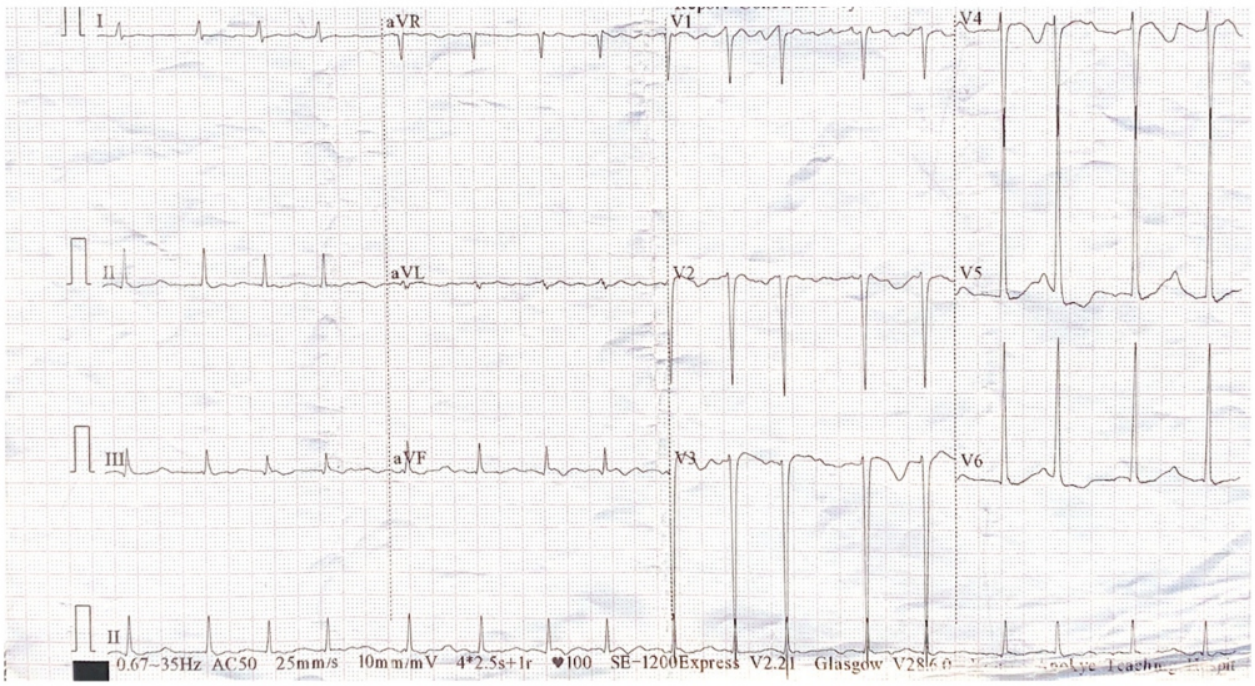

Figure 1. Resting electrocardiogram showing atrial fibrillation with rapid ventricular response, left ventricular hypertrophy, ST/T changes and poor R wave progression.

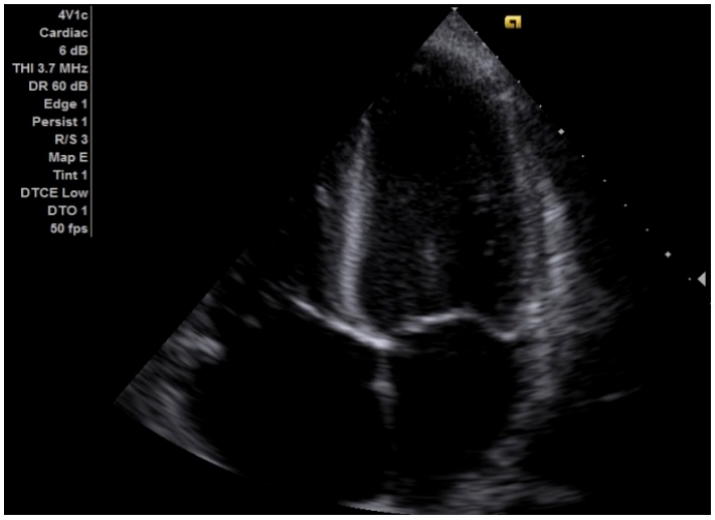

Figure 2. Epical 4 chamber view echocardiogram showing dilated cardiac chambers.

A diagnosis of Graves' disease with thyrotoxic periodic paralysis and heart failure secondary to thyrotoxic cardiomyopathy with atrial fibrillation was made. Patient regained full power in all the limbs with the correction of hypokalemia with 100 mEq of intravenous potassium chloride infused over 24 hours. He was then started on oral potassium (slow K 8 mEq three times daily), furosemide 40 mg daily, sacubitril/valsartan 50 mg twice daily, bisoprolol 5 mg daily, spironolactone 25 mg daily and anticoagulation with warfarin starting at 5 mg at night for the heart failure and atrial fibrillation. He was also put on oral carbimazole 20 mg twice a day and discharged on admission day 4.

At four months post discharge, repeat thyroid hormones were within normal ranges. His atrial fibrillation had spontaneously converted to sinus rhythm. Repeat echocardiography showed improved cardiac dimensions, LVEF had increased from 45% to 51%, right ventricular systolic function also improved (TAPSE increased from 10 mm to 16 mm) and RVSP had reduced from 66 mmHg to 47 mmHg. His heart failure symptoms had resolved and he has since not reported further paralytic episodes. He preferred to continue the thyrotoxicosis treatment with medical therapy rather than surgery or radioiodine therapy.

3. Discussion

TPP is part of a group of diseases known as periodic paralysis characterized by episodes of sudden onset muscle weakness as a result of changes in the excitability of muscles membrane due to abnormal functioning of electrolyte channels which may involve potassium, calcium or sodium channels leading to hyperkalemic, normokalemic or hypokalemic paralysis [17]. The channelopathies are mostly genetic but may also be acquired. TPP is an acquired form of HPP and is differentiated from the familiar type, FPP, by the presence of elevated thyroid hormones which may also be associated with hypomagnesemia and hypophosphatemia [9,17]. Also, FPP is an autosomal dominant condition and there may be other family members with similar disease [10,18].

FPP is a genetic disease and may be due to genetic mutation in CACNA1S which is a dihydropyridine-sensitive calcium channel resulting in type 1 FPP and or SCN4A which is a voltagegated sodium channel in skeletal muscle resulting in type 2 FPP. Also, mutations in the KCNJ2 and KCNJ18 genes that code for inward rectifier potassium channel have also been implicated [18–24].

Although TPP is an acquired disorder, there is growing evidence of genetic involvement. Particularly, patients with Graves' disease who possess the following genetic mutations DCHS2 on 4q31.3, C11orf67 on 11q14.1 and 17q24.3 near KCNJ2 have a higher risk of developing TPP than those without these

mutations [25–28].

TPP by definition is a triad of hyperthyroid state, hypokalemia and paralysis. However, only 45% of patients have overt clinical findings of hyperthyroidism at the time of presentation [29] which can lead to the diagnosis being missed in favor of other closely related differential diagnosis as in the case of this patient at the first presentation. Abnormal electrocardiographic (ECG) findings are found in most patients with TPP either due to the elevated sympathetic activity caused by the excess thyroid hormones or changes related to hypokalemia [29]. Arrhythmias are common with atrial arrhythmias occurring frequently, however ventricular arrhythmias such as ventricular fibrillation can also occur [30]. This patient had atrial fibrillation with ST/T changes on the ECG which may be due to the hypokalemia.

Hypokalemia in TPP results from intracellular shift of potassium. Elevated thyroid hormones increase tissue responsiveness to beta–adrenergic stimulation, which along with insulin and androgens, increases the sodium-potassium ATPase activity in skeletal muscle membranes leading to intracellular shift of potassium, hyperpolarization of the muscle membrane and relative inexcitability of the muscle fibers [18,31]. For this reason, activities that increase insulin or cortisol action such as high carbohydrate meals, alcohol abuse, infections, strenuous exercise and emotional stress are known triggers of TPP [5].

TPP typically occurs in males in the second to fourth decades of life [5] which is consistent with the age and gender of our patient. A few cases however, have been reported in females [13]. Our patient's episodes of paralysis occurred at night which is in keeping with the classic attacks of TPP explaining why this disease was originally described as nocturnal paralysis [32]. The nocturnal presentation of TPP may be explained by the fact that, serum potassium level is influenced by the body's circadian rhythm, such that, serum potassium levels are naturally lower at night and hypokalemia from any cause may be exaggerated at night [33,34]. The pattern of the muscle weakness is symmetrical and involves the proximal muscles commonly affecting the lower extremity muscles [5,10]. However, muscles of the other parts of the body may also be affected including bulbar and respiratory muscles [35].

The diagnosis of TPP involves demonstrating biochemical evidence of elevated thyroid hormones in the context of hypokalemia and paralysis [1,18].

Treatment of TTP involves correcting the underlying hypokalemia with potassium replacement and stabilizing the sodium-potassium ATPase with non-selective beta blockers. The definitive therapy is rendering the patient euthyroid with medical therapy, radioiodine or thyroidectomy [8,10,18,36,37].

4. Conclusions

TPP is increasingly being diagnosed in non-Asians including blacks. It may precede overt clinical manifestation of hyperthyroidism which may pose a diagnostic challenge. However, having a comprehensive approach towards patients with symmetrical paralysis and considering other less common differential diagnosis such as TPP would help ensure early diagnosis and treatment which can avoid complications.

Ethical approval

Patient gave written informed consent for publication of this case report. The case report including the electrocardiogram and echocardiogram images were de-identified to protect patient's privacy and maintain confidentiality.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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Fluoxetine induces oxidative stress-dependent DNA damage in human hepatoma cells

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ABSTRACT

Fluoxetine is a selective serotonin reuptake inhibitor that is a commonly used drug for the treatment of depression and obsessive-compulsive disorders. Despite the positive effects of this drug, it seems to be associated with various side effects. Genotoxicity or DNA damage is an important side effect of some kinds of drugs. To date, the genotoxicity and cytotoxicity of fluoxetine are partially unknown. In the present study, some oxidative stress methods were used, such as ROS, MDA and GSH evaluation methods in HepG2 cells treated with fluoxetine (1–10 μM). A comet assay was used to evaluate the genotoxic effects of fluoxetine, and flow cytometry was used for apoptosis detection in these hepatic cells. Our data have shown that fluoxetine increased MDA and intracellular concentration of ROS significantly ($P < 0.001$), while the amount of GSH was reduced significantly ($P < 0.001$). Our results also indicated that fluoxetine increased the DNA damage of HepG2 cells. The tail percentage of DNA for control cells was 4%, but this percentage was 19%, 28% and 32% for 1, 5 and 10 μM of fluoxetine concentration, respectively ($P < 0.01$ and $P < 0.001$). The flow cytometry results have also shown increases in early and late apoptosis for fluoxetine (13.31% and 9.54%, respectively). In conclusion, the present study has shown that fluoxetine is able to induce oxidative stress-dependent DNA damage. Anyway, more studies are needed to accurately explore the molecular and cellular aspects of fluoxetine

Keywords: fluoxetine; genotoxicity; DNA damage; depression; ROS; apoptosis

1. Introduction

Depression is a common problem and disease in many developed countries, and it is related to lifestyle [1]. There are many therapeutics which have been introduced up to now for some types of depression disorders, such as the tricyclic anti-depressants (TCAs) imipiramine and amtryptiline, which are old drugs with some adverse effects, and selective serotonin reuptake inhibitors (SSRIs), which are newer than TCAs, with fewer adverse effects and better results for anxiety disorders [1]. Monoamine oxidase inhibitors such as tranylcypromine, which is used for some cases of major depression (MD); serotonin-norepinephrine reuptake inhibitors such as venlafaxine, which has almost the same application as SSRIs, with some differences; and some also herbal medicines, which have been applied for depression [1]. Fluoxetine is the most common SSRI, and it serves as the firstline drug for the treatment of MD. MD seems to be a major challenge in medical practice also it can be a social and economic challenge [1]. Due to the high prevalence of MD in human society (10–15% of the population worldwide), fluoxetine is one of the most prescribed drugs [2]. However, its effectiveness is different in different patients with MD. For instance, it has been reported that 60–70% of patients do not experience remission after fluoxetine treatment. However, this type of medication does not show a significant response in 30–40% of patients [3,4].

It is generally accepted that SSRIs, and particularly, fluoxetine, are associated with a large spectrum of

spectrum of side effects. For instance, it has been suggested that, in some cases, fluoxetine treatment causes blurred vision and increased pupil dilation with unknown mechanisms of action [5]. In addition, prolonged treatment with fluoxetine may also increase suicidal tendencies [6]. Moreover, other related studies reported that fluoxetine has unfavorable side effects on the gastrointestinal and central nervous systems [7].

Genotoxicity is generally defined as any type of damage to the whole genome of the organism. Genotoxicity and oxidative stress may affect the regulation and normal activity of cells, contributing to a wide variety of disorders such as malignancy, as well as neurodegenerative diseases [8,9]. For instance, it has been shown that patients who took genotoxic drugs have a higher prevalence of some cancers. A growing body of studies is in support of the ability of genotoxic agents to induce DNA damage through the excess accumulation of reactive oxygen species, i.e., oxidative stress [10]. In oxidative stress circumstances, biological molecules such as DNA, proteins and lipids are damaged.

Reactive oxygen species (ROS) generally affects DNA to break its strands. Prolonged exposure of DNA to ROS leads to a double-strand break and DNA lesion. DNA damage, as one of the most dangerous events of oxidative stress, is detected by sensor proteins such as the MRE11–RAD50–NBS1 complex that transmits the information to some signaling cascade, which eventually induces apoptosis. Lipid oxidation results in the accumulation of malondialdehyde (MDA). Therefore, MDA is served as a biomarker of oxidative stress-induced lipid damage that eventually induces apoptosis [11]. Biological defense against oxidative stress involves increasing of some peptides and proteins as glutathione (GSH) which appears to be increased during oxidative stress.

There are currently few studies on the genotoxicity of fluoxetine in humans, and most of them show inconclusive results. The current study was designed to determine the cytotoxicity and genotoxicity of fluoxetine in HepG2 cells.

2. Materials and methods

2.1. Cell cultures

The human hepatoma cell line (HepG2) was provided by Pasture, Iran. These cells were cultured and incubated in DMEM (Bioidea, Iran) supplemented with 50 mg/L of Pen-strept and 10% fetal bovine serum (Invitrogen, Massachusetts, USA). The cells were also maintained in a humidified incubator containing 5% CO2 at 37 ℃. The culture medium was changed every 48 h, and the cells were also subcultured when their confluence reached 80%.

2.2. DNA damage assessment (comet assay)

The single-cell gel electrophoresis was performed according to the previous study [12]. Briefly, 50,000 cells in each well were cultured for 24 h, followed by treatment with different concentrations of fluoxetine. After 24 h of incubation, the HepG2 cells were harvested by centrifugation at 1,200 rpm at 4 ℃ for 5 min; then they were re-suspended again one time. The cell pellets were finally resuspended in phosphate buffer saline in a cold room for the comet assay. The samples which showed cell viability of more than 70% were further processed for the comet assay. To achieve this goal, 20,000 cells were mixed with 80 µl of 0.5% low-melting-point agarose and added to a glass slide precoated with normal agarose (1%). The samples were then covered by 100-µl low-melting-point agarose, followed by solidification of the gel. The slides were then immersed in lysing solution for 10 h at 4 ℃. Horizontal gel electrophoresis was performed by using a fresh cold alkaline electrophoresis buffer. Electrophoresis was performed at 4 ℃ for 20 min at 15 V (0.8 V/cm) and 300 mA. The slides were then neutralized with 0.4 M Tris buffer at pH 7.5, followed by staining them with ethidium bromide. Each slide was prepared

in triplicate and 100 cells per slide were scored randomly and analyzed by using an image analyzing method (Komet 5.0, Kinetic Imaging, Liverpool, UK). The parameters, such as the DNA tail percentage (% tail DNA = 100 − % head DNA), tail length and tail moment were selected for DNA damage assessment by using Comet software (Kinetic Imaging, Liverpool, UK).

2.3. Intracellular ROS determination

The ROS concentration was evaluated by using 20, 70-dichlorfluorescein-diacetate (DCFH-DA) similar to a previous study [13]. In this study, the cells were treated with different concentrations of fluoxetine, and after the indicated incubation time, 10 mM of DCFH-DA was added to each sample, followed by incubation at 37 ℃ for 1 h. The samples were then washed with Phosphate buffer saline (PBS) and their fluorescence intensity was measured via fluorescence spectroscopy (excitation at 485 nm and emission at 530 nm).

2.4. Intracellular GSH levels assessment

The treated and untreated cells were incubated with monochlorobimane (mBCI, 40 μM) in a staining solution containing 5 mM glucose, 1 mM CaCl2, 0.5 mM MgSO4 and 5 mg/ml Bovine serum albumin (BSA) for 30 min at 37 ℃ under dark conditions. mBCI is a nonfluorescent probe, but it converts to a stable fluorescent adduct with GSH catalyzing the GSH S-transferases. The fluorescent intensity of samples was evaluated at λ = 380 nm for excitation and λ = 460 nm for emission. The fluorescent intensity was calculated as a fold change of control [14].

2.5. Lipid peroxidation assessment

Lipid peroxidation was evaluated according to the spectrophotometric measurement of the product of the reaction of thiobarbituric acid (TBA) and MDA [13]. Briefly, after the indicated treatment, the cells were mixed with 0.5 ml of tricholoroacetic acid (10%, w/v) solution, followed by heating on in a boiling water bath for 20 min. The cells were harvested, and then 1 ml of TBA solution was added to the samples, followed by heating again in boiling water. Finally, the absorbance of the samples was evaluated at 532 nm and the content of MDA was calculated as a fold change of control.

2.6. Flow cytometry assessment of apoptosis

Apoptosis was evaluated by performing annexin V and Propidium iodid (PI) staining as described previously [15]. The cells were cultured at a density of 3 × 105 per well in a six-well plate and incubated with different indicated concentrations of fluoxetine. The cells were washed twice with PBS and stained for 15 min at room temperature with annexin V-FITC and PI. The positive cells for each strain were measured by using the FACS Calibur flow cytometer (Tristar, CA, USA). In flow cytometry analysis, the quadrant quantification is an important issue. Early apoptotic/primary apoptotic cells were annexin V-positive and PI-negative. Late apoptotic cells were determined as both annexin V- and PI-positive; finally, necrotic cells were annexin V-negative and PI-positive. The analysis was performed by using Flow Jo software version 7.6.1 (Tristar, CA, USA).

2.7. Statistical analysis

The tail moment, tail length and percent of DNA in the tail are commonly used in DNA damage assessment. Here, we used these parameters for statistical analysis. One-way analysis of variance (ANOVA) and Tukey's multiple-comparison post hoc tests were done to compare the results of all assays. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Fluoxetine induces genotoxicity

Genotoxicity was measured as the percent tail of DNA and olive tail in treated and untreated cells. The cells were exposed to various concentrations of fluoxetine. As indicated in Figure 1, increasing DNA damage was observed in the HepG2 cells in a dose-dependent manner. The tail percent of DNA for untreated cells was 4%; however, this percentage was 19%, 28% and 32% for 1, 5 and 10 µM of fluoxetine, respectively ($P < 0.01$ and $P < 0.001$). Similarly, the tail moment of DNA was 1, 2, 28 and 35 for the incubation of HepG2 cells with 0, 1, 5 and 10 µM of fluoxetine, respectively.

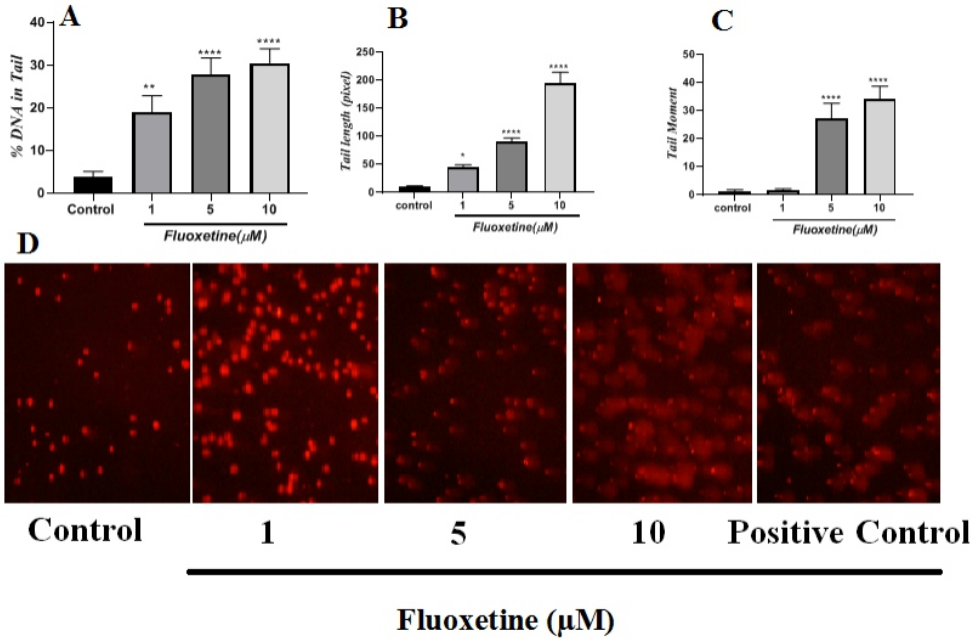


Figure 1. DNA damage in HepG2 cells in response to different concentrations of fluoxetine. A: percentage of tail DNA. B: tail length. C: tail moment. D: representative image of exposed cells. Each value was obtained from three different experiments (mean ± SD). ** and **** were considered as statistically different $P < 0.001$.

3.2. Fluoxetine induces ROS generation

To evaluate the role of ROS in fluoxetine-induced genotoxicity, here, DCFH-DA was used to measure the ROS generation in HepG2 cells in response to fluoxetine stimulation. Incubation with fluoxetine (1–10 µM) for 1 h showed catastrophic increases in oxidant-induced 2', 7'-dichlorofluorescein fluorescence in the HepG2 cells (Figure 2). H2O2-mediated fluorescence emission occurred 1 h after incubation with fluoxetine in the HepG2 cells ($P < 0.0001$), suggesting the involvement of oxidative stress in a concentration-dependent manner.

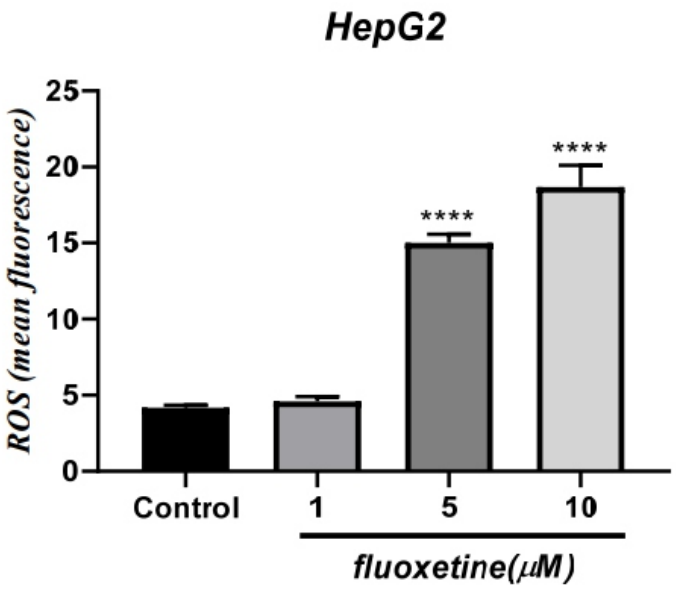


Figure 2. Effect of fluoxetine on ROS generation in HepG2 cells. (****) shows significantly increased results ($P < 0.0001$) as compared to the control group.

3.3. Fluoxetine reduces GSH level

As described in Figure 3, 1 h after treatment with fluoxetine, the intracellular levels of GSH were decreased significantly ($P < 0.001$). This finding was eventually confirmed by an enzymatic assay.

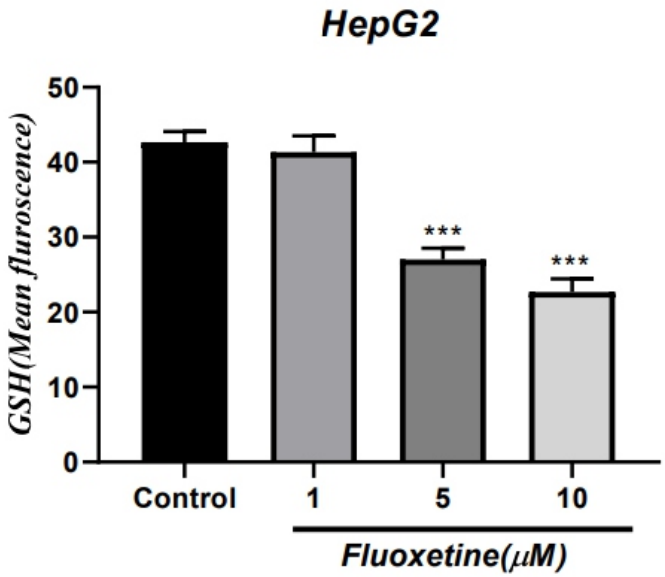


Figure 3. Effect of fluoxetine on the level of intracellular GSH. ANOVA results revealed that fluoxetine significantly decreased the level of GSH. *** stands for statistically significant difference ($P < 0.001$) as compared to the control group.

3.4. Fluoxetine reduces lipid peroxidation

The product of TBA reaction with peroxidized lipid was evaluated. This experiment measures the level of MDA, which is a major product of lipid peroxidation.

As indicated in Figure 4, fluoxetine treatment of HepG2 cells dose-dependently increased the

concentration of MDA ($P < 0.0001$) compared to the control group.

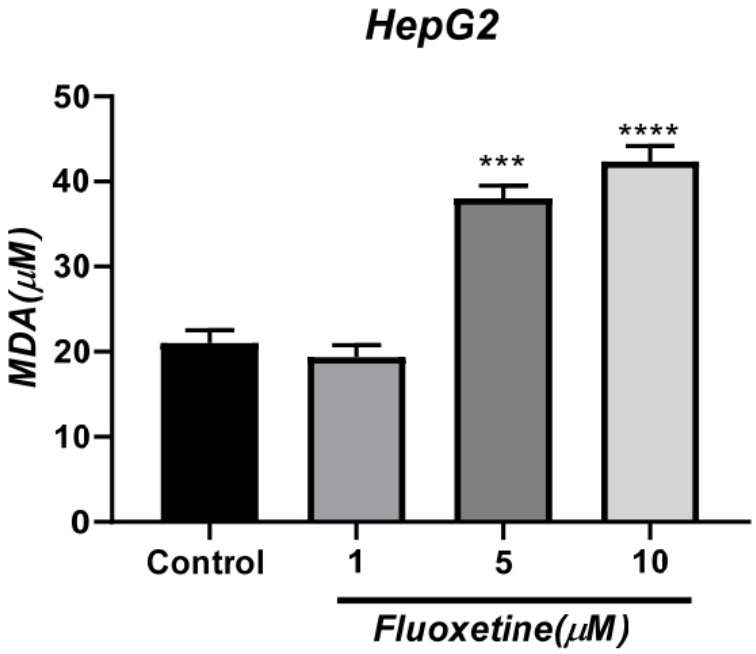
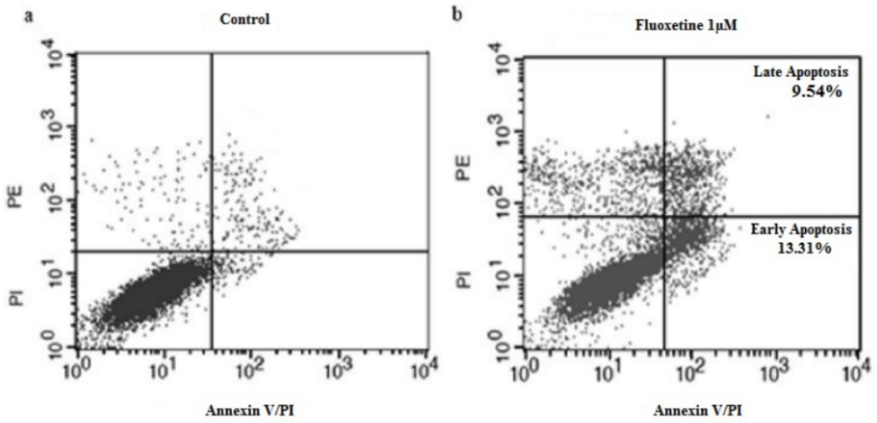


Figure 4. Effect of fluoxetine on MDA levels in HepG2 cells. *** and **** denote statistically significant difference ($P < 0.001$ and $P < 0.0001$, respectively) compared to the control group.

3.5. Fluoxetine induces apoptosis

Due to the morphological change assessment of HepG2 in response to incubation with fluoxetine, flow cytometry analysis was performed. Incubation with three concentrations of fluoxetine (1, 5 and 10 μM) for 24 h resulted in apoptosis induction (Figure 5). As described here, a concentration of 1 μM led to increases in the cells in early and late apoptosis (13.31% and 9.54%, respectively). For the concentration of 5 μM, the percentages of cells in early and late apoptosis were 40.25% and 40.52%, respectively. Finally, for 10 μM, the increases in early and late apoptosis were 25.49% and 48.75%, respectively. The obtained finding confirmed that the death induced by fluoxetine was apoptosis.



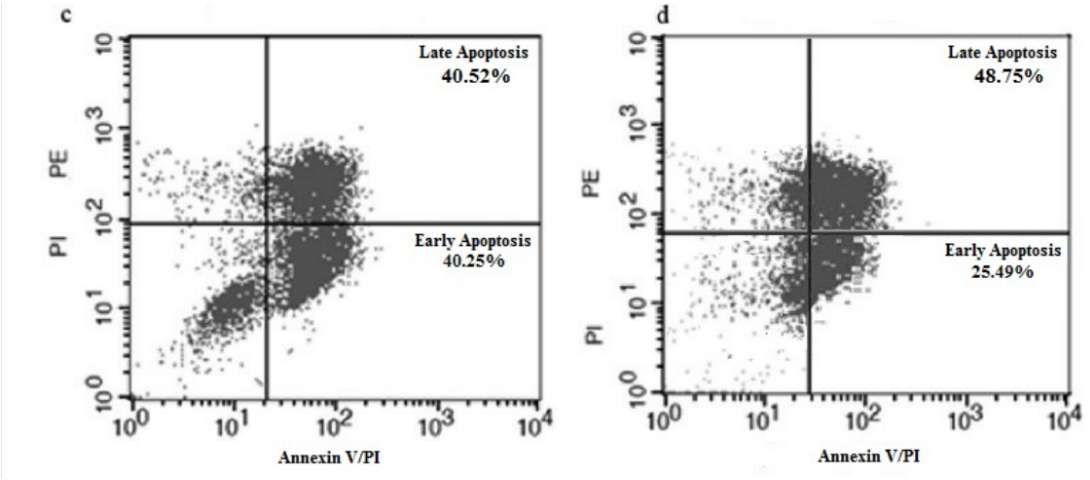


Figure 5. HepG2 cell flow cytometry analysis. (a) Untreated HepG2 cells. (b) HepG2 cells treated with 1 μM of fluoxetine. (c) HepG2 cells treated with 5 μM of fluoxetine. (d) HepG2 cells treated with 10 μM of fluoxetine.

4. Discussion

Fluoxetine is a commonly used pharmaceutical agent purposed to improve the symptoms of some diseases, such as depression, obsessive-compulsive disorder, panic attacks and social phobias. The exact details underlying the fluoxetine mechanism is partially unknown; however, fluoxetine appears to selectively block the reuptake of serotonin in the pre-synaptic space. Although this drug is considered to be safe in adults, some studies indicate that prolonged consumption of fluoxetine may result in endothelial reticulum (ER) stress [16]. Genotoxicity, as one of the main results of ER stress, is a common event in the life of a cell, and it can cause mutation and impaired apoptosis regulation [17,18]. Disruption of the apoptotic pathway is more likely to be involved in a wide variety of cancers and other diseases [19].

Here, our data clearly indicate that fluoxetine treatment resulted in the accumulation of ROS. Given the available knowledge about oxidative stress, we hypothesized that fluoxetine incubation would lead to oxidative stress-induced cell damage. Therefore, oxidative damage to biological molecules such as lipids and DNA were determined. In this regard, our findings also revealed that MDA contents were increased significantly in response to fluoxetine treatment (in a concentration dependent manner). A large body of studies has indicated that, under oxidative stress conditions, cells recruit all defense mechanisms. One of the best-studied natural antioxidant systems is GSH [20]. This tripeptide not only acts as an antioxidant, but it is also involved in the metabolic pathway, redox homeostasis and signaling cascades [21]. For instance, it has been indicated that tert-butyl hydroperoxide exposure of HepG2 cells resulted in a reduction of GSH levels [22]. Our results also showed that fluoxetine induces DNA damage in a concentration-dependent manner.

Previously, Zlatković and coworkers showed that chronic administration of fluoxetine (15 mg/kg/day) induces liver injury. They showed that carbonyl content and MDA increased. However, GSH was decreased significantly, suggesting a potential link between drugs and hepatic oxidative stress [23]. In addition, a human study also showed that MDA and superoxide dismutase were increased after 24 weeks of fluoxetine administration in patients with depression [24]. However, another study revealed that treatment with fluoxetine partially reverses the adverse oxidative stress effects [25,26]. On the other hand, with a glance at the literature, Choi and colleagues indicated that fluoxetine induces ER stress and mitochondrial dysfunction [16]. In addition, Bowie et al. also reported that fluoxetine is an inducer of

of ER stress and autophagy in triple-negative breast cancer cells [27]. Moreover, recently, it has been further confirmed that fluoxetine synergizes with temozolomide to induce ER stress in glioma cells [28]. Other related studies highlighted that ER stress either directly or indirectly resulted in DNA damage and genotoxicity [29,30]. Overall, our study is consistent with previous studies that indicated that fluoxetine, either via oxidative stress or ER stress, induces DNA damage and apoptosis. However, due to a large number of limitations, we could not further investigate the mechanism of action underlying the fluoxetine effects.

5. Conclusions

This study has shown that fluoxetine could induce oxidative stress-dependent DNA damage. So, in clinical practice, more consideration should be given regarding the long use of this drug. However, further precise studies are necessary to further confirm these results. Conflict of interestAll authors declare no conflict of interest regarding the publication of this paper.

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