Assessment of Tumor markers, C-reactive Protein, Cortisol and Total Plasma Peroxides Levels in Uterine Leiomyoma Patients

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Abstract
Objective: The pathophysiology of uterine leiomyoma is yet to be fully understood. This study determined the status of cortisol, C-reactive protein, total plasma peroxide and selected tumor markers in uterine leiomyoma patients.
Materials and Methods: Forty-eight individuals (aged 25-45 years) with uterine leiomyoma (nodules=1-4; size=5-120mm) were recruited for this study. Forty apparently age-matched normal individuals without uterine

leiomyoma served as controls. The patients and controls were selected after confirmation of the status of uterine leiomyoma by ultrasound imaging technique. The plasma levels of total plasma peroxides(TPP), cortisol, carcino-embryonic antigen(CEA), alpha fetoprotein (AFP), carbohydrate antigen 125(CA125) and C-reactive protein(CRP) were determined in them using spectrophotometry, enzyme linked immunosorbent assay and single radial immunodiffusion (Maccini) methods respectively. **Results:** The result shows significantly higher levels of TPP ($p < 0.001$), CRP ($p < 0.001$) and CA125 ($p < 0.002$) in uterine leiomyoma patients when compared with the controls. There were no significant ($p > 0.05$) changes in the plasma levels of cortisol, CEA and AFP in the leiomyoma patients when compared with the controls. Significant ($r = 0.521$, $p = 0.03$) correlation existed between the number of myoma nodules and the levels of CRP in the leiomyoma patients. The size of the nodules correlated significantly ($r = 0.47$, $p = 0.04$) with the plasma levels of TPP. **Conclusion**: Elevated levels of CRP and TPP could indicate oxidative stress and inflammatory response in uterine leiomyoma patients. The induced inflammation and oxidative stress may increase with increase in number and size of the myoma nodules respectively. Higher level of CA125 could be a feature of uterine leiomyoma.

Introduction

Leiomyoma is a benign tumor originating as overgrowth of smooth muscle and connective tissue of the uterus, with ovarian steroid-dependent growth potential. It develops within the wall of the uterus of about 20% of women of reproductive ages when estrogen secretion is maximal (San Marco et al, 1991; Wallach and Vlahos, 2004; Jabbour et al, 2009). They bind 20% more estradiol per milligram of cytoplasmic protein than normal adjacent myometrium (Farber et al, 1972). The striking presence of erythropoietin receptor within myoma tissues contributes to the myoma size (Pollio et al, 2005). Maruo et al (2003) and Maruo et al (2004) reported that progesterone and 17-beta-estradiol stimulate leiomyoma cell growth and survival through up-regulating epidermal growth factor. Uterine leiomyoma that grow as a single (solitary) nodule or clusters (multiple); may present as submucosal, intramuscular, subserosal or pedunculated forms and may range in size from 1 mm to more than 20 cm (8 inches) in diameter (Stewart and Nowak, 1998). The tumor has a clonal karyotype with a complex structural rearrangement involving chromosomes 3, 12, 14, 17, and 22 with malignant potential in 0.2% of cases (Gibas et al, 1988 and Parker et al, 1994). It is also characterized by abnormal nucleotide metabolism, increased deoxyribonucleic acid methylation (Yamagata et al, 2009) and extensive

coagulation necrosis (Katsumori et al, 2002). Certain genes in the fetal life are reactivated in certain tumors as surface proteins of cells in the adults as a result of abnormal or malignant transformation (Carl and Edward, 2001; Gadducci, 2004).

In a normal uterus, there is expression of superoxide dismutase that regulates the constantly generated reactive oxygen species, which serve as a local protector of the uterus against infectious agents (Vural et al, 2012). But in a leiomyoma tissue, activation of infiltrating macrophages generate more free radicals, secrete interleukin-6 and several growth hormones including epidermal growth factor, transforming growth factor-α, transforming growth factor- β, heparin-binding epidermal growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, platelet-derived growth factor, activin and myostatin in myometrium and in leiomyomas (Ciarmela et al, 2011). While the growth factors enhance the growth of the fibrous tissue of leiomyoma, the secreted interleukin-6 induces the hepatocytes to produce C-reactive protein (Jabbour et al, 2009). The excess free radical generation may further attack the DNA and increase the mutation rates, genome instability and loss of apoptosis in the tumor cells (Szabo and Ohshima, 1997). Several reports show that higher level of H_2O_2 is generated from cells that have mitochondrial DNA mutation during tumor development and progression (Bianchi et al, 2001; Mandavilli et al, 2002). The free-radical load and level of unbound iron in the leiomyoma tissue is indicative of the intensification of proliferative activity of the cells of uterine myoma, and one of the risk factors of neoplastic growth (Païlodze and Sanikidze, 2005). To investigate the cellular metabolic changes associated with pathologic proliferation of uterine leiomyoma tissues, the present study paid attention to the determination of total plasma peroxide (TPP), cortisol, C-reactive protein (CRP), carbohydrate antigen 125 (CA125), α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) in uterine leiomyoma patients.

Materials and Methods:
Materials:
Forty-eight individuals (aged 25-45 years) with uterine leiomyoma (nodules=1-4; size=5-120mm) were recruited for this study. Forty apparently age-matched normal individuals without uterine leiomyoma served as controls. The patients and controls were selected by cross sectional study from Oyo, Ogun and Lagos states of Nigeria. The presence or absence of uterine leiomyoma was confirmed by ultrasound imaging technique. The participants were free from metabolic or systemic disorders such as diabetes mellitus, hypertension, liver and renal diseases that could interfere with the findings in this study. None of them was a smoker or on systemic

medications that could affect oxidative stress, inflammation or protein synthesis. This study was approved by the Institutional Review Board, and informed consent obtained from all participants before the commencement of the study. 5ml of venous blood sample was taken from the anticubital vein of each participant into lithium heparin bottle and centrifuged for five minutes at 3,000 revolutions per minute, after which the plasma was separated and stored at -20^0C until analyzed.

Methods:

Determination of total plasma peroxide (TPP):

Determination of total plasma peroxide levels made use of the reaction of ferrous-butylated hydroxytoluene-xylenol orange complex (F0X-2 reagent) with plasma hydrogen peroxide, which yields a color complex that was measured spectrophotometrically at 560mm. H_2O_2 was used as standard. 1.8ml of F0X-2 reagent was mixed with 200µ1 of plasma. This was incubated at room temperature for 30 minutes. 100µM H_2O_2/ L was used as standard. The mixture was centrifuged and the supernatant separated for reading at 560nm (Harma et al, 2003).

Determination of plasma Cortisol, CA125, CEA and AFP:

Five (5) ml of fasting blood sample was collected from each participant into lithium heparin bottle, centrifuged and the plasma stored at -20^0C until ready for analysis. Cortisol, CA125, CEA and AFP were determined by using commercially prepared enzyme linked immunosorbent assay (ELISA) reagents (cat. numbers 24, 94K032, 1110010 and 1107029 respectively) by InterMedical S.R.I. Villanicca (NA) Italy.

Briefly, an aliquot of the plasma (at room temperature) was incubated with enzyme conjugate (corresponding monoclonal antiserum-antibody conjugated with horseradish peroxidase) in the microtiter wells coated with corresponding monoclonal antibody, directed towards a unique antigenic site of either cortisol, CA125, CEA or AFP. After incubation, the unbound conjugate was washed off, the wells drained and the substrate solution added for color development. Sulphoric acid was later added to stop the reaction. The intensity of the color corresponding to the concentration of the analyte was read at 450nm with TECO microplate reader, USA).

Determination of plasma C-reactive protein (CRP):

CRP was quantified by single radial immunodiffusion method as described by Salimonu et al (1978). A volume of an optimally diluted anti-CRP antiserum was mixed with noble agar and poured on glass plate. Wells of equal diameters were cut in the antibody-agar mixture. The wells were

filled with test or standard sera. After incubation, the diameters of precipitin rings were measured using a Hyland viewer with a micrometer eyepiece.

Statistical analysis:

Data were expressed as Mean ± SD. Student's (t) test was used for comparison of leiomyoma patients and controls. Pearsonian correlation coefficient (r) was calculated. Changes were considered significant when p-values were less than 0.05.

Results:

Physical characteristics of uterine leiomyoma patients are similar to the controls (Table 1). In Table 2, the result shows significantly higher levels of TPP ($p<0.001$), CRP ($p<0.001$) and CA125 ($p<0.002$) in uterine leiomyoma patients when compared with the controls. But there were no significant ($p=0.92$, $p=0.13$ and $p=0.35$) changes in the plasma levels of cortisol, CEA and AFP respectively in the leiomyoma patients when compared with the controls. There was a significant ($r=0.521$, $p=0.03$) correlation between the number of myoma nodule and the levels of CRP in the leiomyoma patients as shown in Table 3. The size of the myoma nodules in the patients correlated significant ($r=0.47$, $p=0.04$) with the plasma levels of TPP as shown in Table 4.

Table 1: Physical Characteristics of Uterine Leiomyoma Patients and Controls

	Controls	Leiomy. Patients	p-value	
Age (years)	38.1±10.4	36.0±12.1	10.5	
Weight (Kg)	63.6±8.8	65.4±7.4	10.8	
Height (M)	1.68±0.09	1.65±0.06		8.6
BMI (Kg/M²)	22.8±2.1	23.6±1.8	10.2	
N	40	48		

N= number of subjects used for the study.

Leiomy.= leiomyoma

Table 2: Levels of Total Plasma Peroxides, Tumor Associated Antigens and C-reactive Protein in Uterine Leiomyoma Patients and Controls.

	N	TPP (mmol/L of H_2O_2)	Cortisol ng/ml	CRP (Mg/L)	CEA (ng/ml)	AFP (ng/ml)	CA125 (ng/ml).
Controls	40	11.12±2.8	111.5±82.0	2.5±0.6	2.13±1.6	2.07±3.7	37.5±14.9
Uterine leiomy.	48	17.8±5.7	113.9±77.3	6.4±1.6	1.61±0.9	1.3±2.5	27.3±5.9
p-values		<0.01*	0.92	<0.001*	0.13	0.35	0.002*

*=Significantly different from the controls.

N= number of subjects used for the study.

Leiomy.= leiomyoma.

Table 3: Correlation of TPP, Cortisol, CRP, CEA, AFP, CA125 and Number of Myoma Nodules in uterine leiomyoma patients (N=28)

Groups	r-values	p-values
Number of noddles / TPP,	0.133	0.52
Number of noddles / Cortisol,	-0.283	0.16
Number of noddles / CRP	0.521	0.03*
Number of noddles / CEA	0.031	0.74
Number of noddles / AFP	0.016	0.94
Number of noddles / CA125	-0.333	0.08

* =Significantly correlation.

Table 4: Correlation of TPP, Cortisol, CRP, CEA, AFP, CA125 and Size of Myoma Nodules in uterine leiomyoma patients (N=28)

Groups	r-values	p-values
Size of noddles / TPP,	0.473	0.04*
Size of noddles / Cortisol,	-0.331	0.09
Size of noddles / CRP	0.054	0.78
Size of noddles / CEA	-0.109	0.58
Size of noddles / AFP	-0.133	0.50
Size of noddles / CA125	0.195	0.31

* =Significantly correlation.

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Discussion:

The etiology of uterine leiomyoma is poorly understood, but available information revealed that it is a slow growing non-cancerous macrophage infiltrated tumor, characterized by abnormal nucleotide metabolism, increased DNA methylation (Yamagata et al, 2009), loss of cyclin G1 expression, extensive coagulation necrosis (Kwon et al, 2008), hypercellularity, nuclear atypia, intravascular growth (Prayson and Hart 1995) and chromosomal aberration (Stern et al, 1992). Sadlonova et al. (2008) reported metabolic dysfunctions such as abnormal membrane protein expression in leiomyoma patients. One of the main findings of the present study is that plasma level of CA125 increased significantly in leiomyoma patients. This study seems to corroborate the report of Tsao et al (2007) that demonstrated elevated levels of CA19.9 and CA125 in leiomyoma tissues. (Ghaemmaghami et al, 2007) also reported higher levels of CA125 in patients with other gynecologic diseases without malignant conditions. The possible changes already reported in the metabolic activities of uterine leiomyoma tissue could therefore account for the increased level of CA125 in our uterine leiomyoma patients. This study did not demonstrate significant change in the level of CEA. Meanwhile, higher level of plasma CEA had been consistently reported in certain gynecologic malignancies (DiSaia et al, 1975). Since inflammatory cytokines (i.e. IL1 and IL6) that induce the synthesis of CRP have suppressive effects on the synthesis of albumin and AFP, it could be hypothesized in this study that insignificant change in the level of AFP observed in this study may be associated with increased level of CRP observed in the leiomyoma patients. This study seems to agree with the report by Philippoussis et al (2004) that patients with endometriosis did not show significant change in the serum level of AFP.

Cells of the endometrium generate reactive oxygen species as its local protectors against pathogens. But the free radicals are regulated by superoxide dismutase also released into the uterus (Vural et al, 2012). Since Bianchi et al. (2001) and Mandavilli et al. (2002) reported that higher level of hydrogen peroxide was generated from cells that have mitochondrial DNA mutation during tumor development and progression, the deregulated level of TPP observed in our leiomyoma patients could be due to enhanced free radical generation in the macrophage infiltrated myoma tissues. It could also be due to increased surface area of larger myoma that causes enhanced prostaglandin activity needed for myometrial contraction; to maintain homeostasis at menstruation, cellular injury, inflammations or vasoconstriction-induced hypoxia during menstruation (Stewart and Nowak, 1998). Our findings are consistent with previous studies reporting elevated oxidative stress in leiomyoma patients (Punnonen et al, 1993 and Ghiselli et al, 2000). Thalmann et al. (2000) and **Kumar** et al. (2008) also observed

increased free radical generation and metabolic dysfunctions in tumor cells when compared with normal cells. This higher free radical load has been associated with higher malondialdehyde in the uterine myoma patients (Chiou and Hu, 1999). To the knowledge of the authors, this is the first study that shows significant positive correlation between the size of the myoma nodules and plasma levels of TPP. This excessive generation of hydrogen peroxides in relation to the size of the tumor may contribute to the progressive DNA damage and genomic instability that could facilitate subsequent progression to malignancy, as previously reported by Nelson et al (2004). It has also been reported that such elevated levels of H_2O_2 and superoxide anion could induce mitogenesis, cell proliferation and malignant transformation of several mammalian cell types (D'Souza et al, 1993).

One of the novels of this study could be the positive correlation observed between the number of myoma nodules and plasma level of CRP in our leiomyoma patients. This could be an expression of level of inflammatory response in the patients. Previous reports show that activated macrophages secrete several cytokines (i.e. interleukin-6) capable of initiating the production of CRP and other spectrum of acute phase proteins by the hepatocytes (Jabbour et al, 2009). The physiological roles of the CRP include regulation of immune responses, mediation and inhibition of inflammation, acting as transport proteins for products generated during the inflammatory process, acting as opsonins, prevention of apoptosis, activation of complements, binding of cellular remnants like nuclear fractions (Rienhoff, 1990), scavenging free haemoglobin and radicals, repairs and remodeling of tissue. Increased level of CRP observed in this study corroborates the findings of Hemilä et al (1987) that CRP increased significantly in leiomyoma patients. Therefore, elevated CRP in uterine leiomyoma patient may suggest possibility of chronic inflammation. It may also be a protective immunologic measure, employed by the hepatocytes to reduce the risks associated with inflammation (Jabbour et al, 2009).

The tissue level of cortisol is controlled by 11-beta hydroxysteroid dehydrogenase -1 (11-beta HSD-1) that catalyzes the conversion of biologically inert cortisone to biologically active cortisol, while 11-beta HSD-2 utilizes the cofactor NAD+ to convert cortisol to cortisone. Antagonistic effects of cortisol on 17-beta-estradiol in the rat uterus (Rhen et al, 2003) indicate that cortisol inhibits leiomyoma growth induction of estradiol. The insignificant change observed in the level of cortisol in our uterine leiomyoma patients could be due to local regulation of cortisol biosynthesis in reproductive tissue, through the oxidative activity of 11-beta HSD that catalyzes both oxidative and reductive reactions of glucocorticoids (Michael et al, 2003).

Conclusion: Elevated levels of CRP and TPP could indicate oxidative stress and inflammatory response (respectively) in uterine leiomyoma patients. The induced inflammation and oxidative stress may increase with increase in number and size of the myoma nodules respectively. Adjuvant antioxidant and anti-inflammatory therapies may be needed to avert the consequences of oxidative stress and chronic inflammation that could lead to malignant transformation of myomas. Higher level of CA125 could be a confirmation that uterine leiomyoma is a tumor.

Competing interests:
The authors declare that they have no competing interests.

Authors' contributions:
MA, AA SA and OJ designed the study, MA, AA and OJ and AO did the analysis, and all authors prepared and approved the final manuscript.

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