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Liver iron overload induced by tamoxifen in diabetic and non-diabetic female Wistar rats

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Abstract Tamoxifen (TX), a drug used in the treatment of breast cancer, may cause hepatic changes in some patients. The consequences of its use on the liver tissues of rats with or without diabetes mellitus (DM) have not been fully explored. The purpose of this study was to evaluate the correlation between plasma hepatic enzyme levels and the presence of iron overload in the hepatic tissue of female Wistar rats with or without streptozotocin-induced DM and using TX. Female rats were studied in control groups: C-0 (non-drug users), C-V (sorbitol vehicle only) and C-TX (using TX). DM (diabetic non-drug users) and DM-TX (diabetics using TX) were the test groups. Sixty days after induced DM, blood samples were collected for glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST) alkaline phosphatase (ALP) and bilirubin measures. Hepatic fragments were processed and stained with hematoxylin and eosin, Masson's trichrome, Perls. The hepatic iron content was quantified by atomic absorption spectrometry. AST, ALT and ALP levels were significantly elevated in the DM and DM-TX groups, with unchanged bilirubin levels. Liver iron overload using Perls stain and atomic absorption spectrometry were observed exclusively in groups C-TX and DM-TX. There was positive correlation between AST, ALT and ALP levels and microscopic hepatic siderosis intensity in group DM-TX. In conclusion, TX administration is associated with liver siderosis in diabetic and non-diabetic rats. In addition, TX induced liver iron overload with unaltered hepatic function in non-diabetic rats and may be a useful tool for investigating the biological control of iron metabolism.

Keywords Liver · Hemosiderin · Siderosis · Iron metabolism · Diabetes mellitus

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Introduction

Since 1970, TX (tamoxifen, trans-isomer of 1-(ρ-β-dimethylethanamine)-1,2-diphenyl-1-butenyl citrate) has been widely used in the treatment and prevention of recurring breast cancer (Cole et al. 1971). It is a non-steroid antiestrogenic drug of proven effectiveness against breast carcinomas that express estrogen receptors (Bonnani and Veronesi 1999; Cole et al. 1971).

However, TX may alter hepatic enzyme levels, above all, ALT and AST (Lox et al. 1998). Elefsiniotis et al. (2004) observed, in women with breast cancer and hepatic steatosis, that this drug induced hepatotoxicity in a large proportion of cases, especially when associated to high body mass index and high levels of glucose and serum lipids.

There are few studies reporting the impact of TX use in women with DM on hepatic enzymes and possible histological liver alterations.

Despite enormous clinical success and increased survival rates, some evidence suggests that long-term TX use may induce a wide spectrum of hepatic lesions such as periportal hepatitis and cirrhosis (Maruyama et al. 1995), macrovesicular steatosis and steatohepatitis (Pinto et al. 1995).

Tamoxifen is related to aneuploidy induction and DNA damage in animal hepatocytes (Sargent et al. 1994; Kim et al. 2006), and human hepatocytes (Kim et al. 2005). It is believed that the toxic effect of tamoxifen on hepatocytes is due to the appearance of reactive metabolites during their metabolism (Park et al. 2005).

The investigation of other possible hepatic aggressions resulting from TX use, particularly iron overload (siderosis), has been equally little studied. Hepatic iron overload is often seen in acquired and inherited disorders (Bacon and Britton 1990; Bulaj et al. 2000; Malina et al. 2005).

Porphyria cutanea tarda, a disorder characterized by photosensitive dermatosis and iron overload, is another association observed in several TX-treated patients with breast cancer (Bulaj et al. 2000; Malina et al. 2005). Iron overload is one of the factors that triggers the clinical manifestations of this disease (Sampietro et al. 1999).

The precise mechanism of the hepatocelular damage caused by iron overload remains unclear, but iron has a catalytic effect on free radical generation that promotes lipid peroxidation in the hepatocytes responsible for the rupture of membranes, causing loss of organelle integrity (Bacon et al. 1985).

Many breast cancer patients that use this drug are also diabetic. There is a report of increased insulin need in diabetic patients using TX (Kurt et al. 2005). The purpose of this study was to determine the presence or not of hepatic damage produced by daily doses of TX proportionally equivalent to humans in normal euglycemic rats and in streptozotocin (STZ)-induced diabetic rats. In positive cases, hepatic lesions were verified quantitatively and qualitatively. Biochemical analysis of bilirubin, hepatic iron content, AST, ALT and ALP were also performed to find a correlation with TX use and to compare between diabetic and non-diabetic groups.

Material and methods

Animals

Twenty-five Wistar rats (220–300 g), aged 16–20 weeks with regular estrogen cycle, verified by vaginal cytology, were studied (Duarte et al. 2005). During the experimental period the animals were maintained under standard temperature conditions and subjected to a 12 h/12 h light/dark cycle and fed water and standard ration (Labina®, Purina, São Paulo, Brazil), containing 180 mg/kg of iron ad libitum, according to the specifications described by Reeves et al. (1993). The animals were supplied by the Department of Surgery of the Federal University of Rio Grande do Norte. Routine hygiene was performed, including the cleaning of plastic cages and daily change of the tap water throughout the experimental period. The animals were treated according to the "National Research Council Guide for the Care and Use of Laboratory Animals". All procedures described in this study were carried out after approval by the institutional Ethics Committee, under registration number 83/85.

Protocols

The animals were randomly divided into five groups of five rats each. No drug was administered to the C-0 group (control group). The C-V group used only

vehicle (10% sorbitol); the C-TX group used TX and vehicle; the two diabetic groups were composed of the DM group (no TX or vehicle) and DM-TX group (using TX and vehicle). The ingestion of sorbitol does not interfere on glycemia levels (Brunzell 1978). In the present study, sorbitol was used as a sweetener to facilitate the ingestion of the drug without raising glucose levels. The weights of all the animals were measured on the first day of the experiment and on the day they were sacrificed.

Induction of diabetes mellitus

Experimental diabetes was induced in the animals by a single intra-peritoneal injection of the pancreatic β-cell toxin STZ (streptozotocin, Sigma Chemical Co., Spain), dissolved fresh in sterile citrate buffer 0.01 M, pH 4.5, at a dose of 45 mg/kg body weight. Equal volumes of this vehicle were injected into the control rats. At day 0, i.e., 5 days after induction, blood was collected by tail bleeding and assayed using the glucose-oxidase method (Advantage-Boehringer Mannheim, Roche, USA), and glucose levels were monitored throughout the study to determine the hyperglycemic state of the animals. Body weight evolution of the animals was monitored at 15-day intervals throughout the 60 days after the onset of diabetes at day 0 (Duarte 2005).

Procedures for administering TX

Suspension of TX (tamoxifen, Zeneca Pharmaceuticals, USA) at a concentration of 25 mg/mL was prepared in aqueous solution of 10% sorbitol, 1% of tween 20 and 5% of carboximethylcellulose. Group C-TX and DM-TX animals were treated daily with TX, 0.3 mg/kg by gavage, throughout the 60-day period (Silva 2005).

Blood collection and routine biochemical analysis

All the animals were killed by cervical dislocation without anesthetic to avoid any interference with the assay and a blood sample was obtained from cardiac puncture. In order to prevent any possible daily cyclic variations in the measurements, the animals were always sacrificed between 7:00 and 9:00 am. Serum glucose, ALT, AST, ALP and bilirubin levels were measured using routine methods (Labtest, Brazil). ALP [E.C. 3.1.3..1.] activity was measured with 4-nitrophenylphosphate as substrate and is expressed as units (U), with one unit corresponding to the hydrolysis of 1 mmol of 4-nitrophenylphosphate per minute at 37°C. ALT activity was measured with L-alanine and ketoglutarate as substrate, malate dehydrogenase and coenzyme NADH, and is expressed as U/L, with a unit corresponding to the hydrolysis of substrate per minute at 37°C. AST activity was measured with L-aspatate and ketoglutarate as substrate, enzyme malate dehydrogenase and coenzyme NADH, and is expressed as U/L, with a unit corresponding to the hydrolysis of substrate per minute at 37°C. All serum measurements were performed with a Cobas Mira analyzer (Roche diagnostics, Swiss) in triplicate (Bergmeyer 1978).

Tissue sample processing

For light microscopic analysis, liver tissue samples of the right and left lobes were obtained after sacrificing the animals and fixed in 10% buffered formalin (pH 6.8) and processed as described by Niemi and Korhonen (1972). The histological slides obtained were stained with hematoxilin and eosin (H&E), Masson's trichrome for collagen and Perls' Prussian blue method to detect iron deposits (Masson 1929; Stevens and Chalk 2001). Liver tissue samples were frozen at −80°C in preparation for a subsequent biochemical measure of iron.

Quantitative analysis of iron deposits in liver tissue

The Prussian blue staining was quantified in the liver tissue using a digitalized system and an image analyzer. The total area of microscopic fields in the histological sections stained by Perls' histochemical technique was observed using Olympus BX-45 (Olympus, Japan) triocular optical microscope. The images were captured with a digital camera (Samsung, Korea) and processed using Image Pro-plus software, version 3.0 (Media Cybernetics—LP, USA). The digitalized field was divided by the software into image units denominated picture elements or pixels, with definite coordinates. Four digitalized images of the histological sections of each animal were obtained and examined for quantifying hemosiderin pigment, whose intensity (iron deposits) was determined by the arithmetic mean of

the number of hemosiderin pixels for the four separate fields, each composed of 303,360 pixels.

The iron content of the frozen liver samples was determined according to the technique described by Olynyk et al. (1994). The specimens were dried in an oven for 24 h at 65°C, weighed, digested in acid medium and the liver iron content was determined by atomic absorption spectroscopy (Spectra AA200, Varian, Australia) and results expressed in μg/g.

Statistical analysis

Student's *t*, Kruskal–Wallis *H*, Tukey and Dunnett were the statistical tests used to verify differences between the groups in relation to glucose**,** ALT, AST and ALP values, as well as for counting hemosiderin pixels for each field of 303,360 pixels and the dry weight measure of the iron extracted from the liver tissue.

Results

Animals

Animals with blood glucose concentrations higher than 250 mg/dL were considered to be diabetic. In addition to hyperglycemia, the animals showed clinical signs of diabetes such as polyphagia, polydipsia, polyuria and body weight loss. The C-V and C-TX groups injected with vehicle without STZ, maintained normal blood glucose levels.

Diabetic rats showed significantly elevated blood glucose concentrations ($P < 0.05$) (Table 1) compared to age-matched controls C-0, C-V, and C-TX over the course of the experiment. The serum total bilirubin levels of both groups using TX were normal.

Biochemical analysis

Table 1 also shows the serum levels of ALT, AST and ALP. The diabetic groups (DM and DM-TX) always had higher mean values than those of non-diabetics (C-0, C-V, and C-TX), regardless of the parameter assessed. Mean ALT and AST levels were always above 200 U/L (Table 1), with significant differences ($P < 0.005$) when comparing groups DM and DM-TX with the remaining groups (C-0, C-V, C-TX), except when compared to each other. ALP revealed serum levels above 250 U/L in groups DM and DM-TX and always greater than the non-diabetic groups (C-0, C-V, C-TX) (Table 1).

Table 1 Descriptive statistics and statistical tests related to siderosis counts (per field of 303,360 pixels) and biochemical measures of Glucose, ALT, AST, ALP and Bilirubin

Groups	C-O	C-V	DM	C-TX	DM-TX	*P*-values[1]	*P*-values[2]
Variables							
Siderosis	–	–	–	182 ± 2.0	186 ± 1.7	0.004*	–
Glucose (mg/dL)[4]	125 ± 11.6 [a]	121 ± 6.3 [b]	366 ± 90.1[ab]	90 ± 7.0 [ab]	391 ± 183.6	0.021*	0.000*
ALT (U/L)[4]	42 ± 4.8 [a]	41 ± 4.3 [b]	248 ± 79.2 [c]	39 ± 7.4 [abc]	205 ± 131.7	0.048*	0.001*
AST (U/L)[4]	59 ± 10.5 [a]	43 ± 3.1 [b]	250 ± 79.9 [c]	42 ± 7.9 [abc]	209 ± 131.3	0.047*	0.000*
ALP (U/L)[4]	74 ± 13.6 [a]	161 ± 89.7 [b]	420 ± 295.1	95 ± 37.3 [c]	677 ± 185.7 [abc]	0.000*	0.001*
Iron (μg/g)[3]	680 ± 311[a]	–	–	1196 ± 572.1[a]	1073 ± 293.1	0.206	–
Bilirubin (mg/dL)[4]	0.82 ± 0.04[a]	0.83 ± 0.05	1.07 ± 0.22	0.86 ± 0.09	1.24 ± 0.40[a]	0.103	0.020*

C-0: control group using no drug whatsoever. C-V: group using only sorbitol. DM: diabetic group using no drug whatsoever C-TX: non-diabetic group using tamoxifen and DM-TX: diabetic group using tamoxifen

Source: Experimental research—Authoŕs data

Mean ± Standard Deviation

Siderosis not observed under optic microscope in groups C-0, C-V and DM.

[1] Student *t*-test *P*-value for comparisons of the means of non-diabetic (C-TX) and diabetic (DM-TX) groups

[2] *P*-value by Kruskal–Wallis *H* test

[3] Values of this variable followed by at least one equal letter differ significantly at a level of 5% using Tukey's test

[4] Values of this variable followed by at least one equal letter differ significantly at a level of 5% using Dunnett's test

Quantitative analysis of iron deposits in hepatic tissue

Microscopic assessment of hepatic tissue samples stained by H&E and Masson's trichrome did not reveal specific alterations, underscoring the absence of abnormal collagen deposition or steatosis. Iron overload in the hepatic tissue histochemically assessed using Perls' Prussian blue staining method, was present exclusively in groups DM-TX and C-TX (Fig. 1 and Table 1). Therefore, group DM-TX had higher mean liver iron deposits than those found in group C-TX, (Table 1) considering the histologically observed siderosis. In animals where iron was detected (C-TX and DM-TX), there was no evidence of a preference for specific liver zones, given that it was found uniformly in both hepatocyte and Kupffer cells (Fig. 2).

Biochemical measures of iron in the dry liver weight were taken of individuals in groups C-TX and DM-TX whose histopathology showed siderosis. The iron values of group C-TX were higher than those of group DM-TX, but not statistically significant (Table 1).

Discussion

The use of TX has provided clinically successful treatment of breast cancer. On the other hand, some evidence suggests that prolonged TX use may induce a wide range of hepatic changes. The natural history, progression and possible clinical outcomes of these lesions still need to be more thoroughly studied.

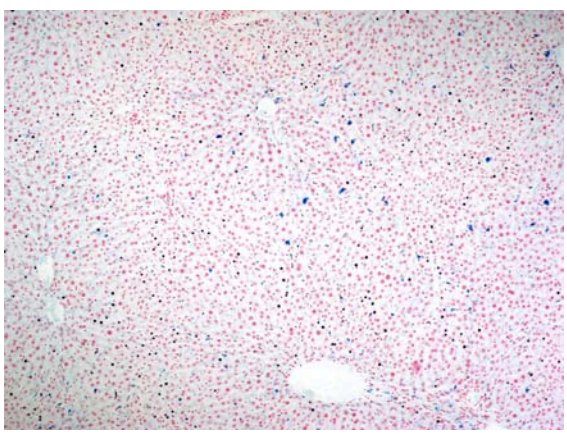

Fig. 1 Liver microscopy of group DM-TX (×100): Section stained by Perls' Prussian blue. At this magnification, the iron deposits are visible as blue areas and hepatocyte staining details are not as evident as they are in Kuppfer cells

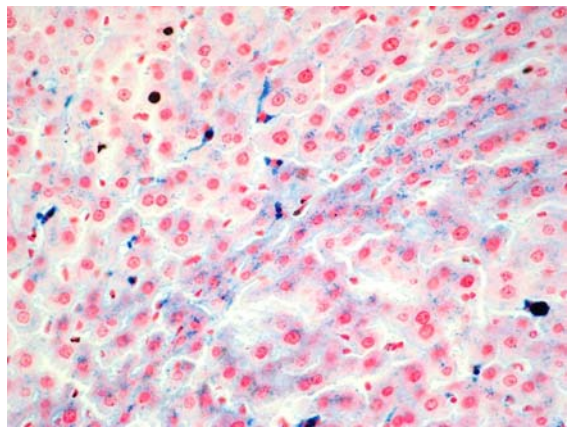

Fig. 2 Liver microscopy of group DM-TX (×200): Section stained by Perls' Prussian blue. The iron deposits are stained as blue granules

Besides the evidence of microscopic liver lesions, some authors have reported changes in hepatic enzyme biochemical profile in the plasma of patients treated with TX (Morgan et al. 1976). The population using TX exhibits characteristic clinical profile, composed for the most part of menopausal women with secondary hypertriglyceridemia and mainly type 2 DM (Morgan et al. 1976; Milionis et al. 2001). This fact raises speculation about its potential synergism in provoking clinically significant hepatic changes.

Our results, in relation to the histopathological study of the livers of diabetic or non-diabetic TX-treated rats, showed that there was no microscopically identifiable alteration in acinar architecture, notably steatosis, hepatocytic necrosis and/or abnormal collagen deposition after TX use for 60 days (Figs. 1, 2).

This histochemical technique allows a detailed analysis of the cell and acinar location of the iron deposits (Brunt 2005). In animals where iron was histologically detected (C-TX and DM-TX) there was no predominance of deposits in specific hepatic zones, with iron found both in hepatocyte and Kupffer cells (Fig. 2). The validity of this histochemical method, as a semi-quantitative measure of liver iron content, is based on the fact that it favors the simultaneous histopathological study of associated hepatic lesions while maintaining a narrow correlation

with biochemically determined liver iron (Ortega 2005). In this sense, our results corroborate those of Ortega (2005), since the biochemical iron measures of liver dry weight of the groups with siderosis (C-TX and DM-TX) had elevated levels, confirming the results obtained in the histopathological study.

The absence of hemolytic activity, verified by the normal serum bilirubin levels, indicates that this iron accumulation does not derive from heme. The iron levels in dry weight liver tissue confirmed the results obtained by Perls' technique in histological sections. Therefore, this technique proves the presence of iron overload in the liver of animals that used TX, diabetic or not (Table 1).

The mechanisms by which the hepatocellular accumulation of iron can cause liver disease have already been studied (Britton 1994). It is suggested that iron promotes the release of fibrogenic substances, which activate hepatic stellate cells—responsible for the synthesis of collagen and other extracellular matrix components during chronic liver disease (Trinder et al. 1998). This statement is based on studies involving genetic expression of collagen, indicating that iron deposit in hepatocytes is necessary for the expression of these genes (Gualdi 1994). Ferritin is the main intracellular protein responsible for capturing excess free iron. It does so to minimize free radical generation in reactions where this ion acts as a catalyzer. In the present study, we defend the hypothesis that this accumulation may be a consequence of free radical formation during TX metabolism. In liver cells submitted to oxidative stress, transcription activation of the ferritin heavy-chain gene (ferritin H) occurs through JunD transcription factor activity (Tsuji 2005). This is a cell protection mechanism that avoids dangerous iron ion activity in oxidative stress induction.

Normal iron metabolism is highly regulated and is extremely important in maintaining cellular functions. In this process, the liver plays an important role in the synthesis of proteins such as transferrin and ceruplasmin and in the regulation of plasma iron levels, through the production of plasma peptide hepcidin (Anderson 2005) which regulates intestinal absorption. Hepcidin interacts directly with ferroportin (FPN), the only iron exporter known in mammals, and is expressed in macrophages, hepatocytes and enterocytes (Atanasiu 2007). An interference in the regulating mechanism of hepcidin may result in abnormal iron deposits such as in hereditary hemochromatosis, where its expression is unduly diminished (Fleming 2005). The hypothesis of the interference of drugs such as TX on this homeostatic mechanism cannot be ruled out, and should be the object of future studies. Recent studies have shown that lipid peroxidation, resulting from iron-mediated free radicals damages cell membrane polyunsaturated fatty acids, presumably involved in hepatic lesion pathogenesis (Bacon et al. 1985). It has been shown that lipid degradation products also stimulate collagen production in hepatic stellate cell culture and in human fibroblasts (Maher 1994). These products may also activate hepatic stellate cells and stimulate the production of transforming growth factor β (TGF-β) through Kupffer cells (Leonarduzzi 1997).

Some studies have referred to the occurrence of oxidative stress in hepatocytes as a result of TX metabolism, accompanied by the formation of hydrogen peroxide (H_2O_2) and lipid degradation products (Parvez et al. 2006). Another study showed the formation of oxygen reactive species and apoptosis induction mediated by calcium influx in HepG2 cells treated with TX (Lee et al. 2000).

The use of TX in animals with induced DM (DM-TX) caused ALP increase when compared to the diabetic group not using TX (DM) (Table 1) but this difference was not statistically significant. The results found by Tibi et al. (1988) showed that liver ALP was significantly higher in diabetic patients compared with the control group and bone ALP showed no significant difference between the diabetic patients and control group. In group DM, ALT and AST values were significantly higher than those of group C-TX. These results lead us to conclude that TX did not affect enzyme levels, but the presence of diabetes mellitus can influence the enzyme results.

There are still no reports in the literature showing hepatic siderosis in women using TX. Nevertheless, we recommend that more clinical studies should be performed to determine if the changes reported also occur in humans with or without DM in order to prevent clinically significant lesions. In addition, the TX administration to non-diabetic Wistar rats could be adopted as a new experimental model for studying the biological control of iron metabolism, offering the possibility of new therapeutic approaches to iron metabolism disorders, especially those related to iron absorption and mobilization (Ganz 2005).

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