Anthracycline-induced cardiotoxicity, a pathophysiology based approach for early detection and protective strategies

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CHAPTER 3

Disturbed iron metabolism due to anthracycline-based chemotherapy in early stage, surgically cured female breast cancer patients

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ABSTRACT

INTRODUCTION Iron-catalyzed free radicals seem to play a role in anthracycline-induced cardiotoxicity and may lead to organ damage and dysfunction. The aim of this study was to evaluate iron metabolism in breast cancer patients who received adjuvant doxorubicin/cyclophosphamide treatment (AC).

PATIENTS AND METHODS We included 39 female breast-cancer patients (median age 47), scheduled to receive intravenous AC-chemotherapy. Iron status [total iron, transferrin, ferritin, latent iron binding capacity (LIBC) and non-protein bound iron (NPBI)] was studied during the first course of chemotherapy. Samples were taken prior to, immediately and 2:30 hrs after the doxorubicin infusion (total iron, LIBC and NPBI) and at 24 hrs after completion of the chemotherapy course. Additional measurements (at baseline and 24 hrs) were done during the subsequent chemotherapy courses and at 1 and 4 months after completion of the entire chemotherapy treatment.

RESULTS Immediately after the first administration of doxorubicin NPBI increased by 65.7% (95%CI: 23.5 to 122.3%) and returned to baseline at 24 hours. In parallel, total iron increased with 187.1% (95%CI: 153.7 to 225.0%) at 24 hours, accompanied by an almost total saturation of transferrin. Ferritin levels increased gradually over baseline, and were 79.1% (95%CI: 37.1 to 33.9%) higher at baseline of the fifth course.

DISCUSSION AND CONCLUSION This study shows that a single intravenous dose of doxorubicin immediately results in an increase of highly toxic NPBI in early stage breast cancer patients and this suggest that that NPBI may be, at least in part, responsible for the toxicity caused by doxorubicin.

INTRODUCTION

Anthracyclines are used in the treatment of several cancers because of their ability to inhibit topoisomerase II.(1) They are also known to facilitate the formation of free radicals in the presence of (non-protein bound) iron, which are supposed to be responsible for the (cardio)toxic side effects of anthracyclines.(1-4)

There seems to be consensus that under physiological conditions non-protein bound iron (NPBI, or sometimes referred to as non-transferrin bound iron) is not present extracellularly as iron is tightly bound to transport- and storage proteins, such as transferrin (serum) and ferritin (intracellular).(5) However, in case of iron overload, which may occur in hemochromatosis, dialysis, hemolytic anemia's and after certain (mostly high-dose) chemotherapy regimens the presence of NPBI has been reported. (6-11) This suggests that the binding capacity of the transport- and storage proteins does not suffice under these pathological conditions. NPBI in its ferrous form (Fe^{2+}) is highly reactive and capable to catalyze the Haber-Weiss reaction in which hydroxyl radicals are formed, resulting in lipid peroxidation, DNA damage, and eventually apoptosis.(5,12-14) It can be envisaged that in the case of anthracycline-induced cardiotoxicity, NPBI can even be more harmful as the heart has relatively low levels of antioxidants and (excess) iron is able to form a stable complex with doxorubicin (DOX), which easily undergoes self-reduction to form a semiquinone free radical of DOX.(3;12) In addition, *in vitro* studies have shown that anthracyclines deregulate intra-cellular iron metabolism and iron trafficking pathways, thereby aggravating the effects of (intracellular) iron overload.(13;14)

Although iron metabolism has been investigated in cancer patients receiving high doses of chemotherapy, effects in (surgically tumor-free) cancer patients receiving lower doses of anthracyclines have not been studied. Therefore, we performed a study to evaluate the effects of the combination of the anthracycline doxorubicin and cyclophosphamide (AC) on iron metabolism in female breast cancer patients who underwent adjuvant treatment with AC chemotherapy for early stage breast cancer.

METHODS

Patient population and study protocol

The patient population consisted of early-stage female breast cancer patients who underwent adjuvant treatment with a combination of doxorubicin (DOX) and cyclophosphamide chemotherapy. Main exclusion criteria included prior or concomitant use of cardiotoxic medication, distant metastases, a history of other malignant disease, a life expectancy of less than one year, pre-existing cardiovascular diseases and elevated transaminases above 3 times the upper limit of normal.

Eligible patients were scheduled for four or five (depending on the institutional guideline) three-weekly courses of intravenous (IV) doxorubicin (60mg/m^2 over 15 min) and cyclophosphamide (600 mg/m^2 over 15 min).

The medical ethical committee of Leiden University Medical Center (LUMC) approved the study protocol before inclusion of the first subject. All subjects gave written informed consent before participation.

Study procedures and measurements

Before the first course of chemotherapy concentrations of total iron, latent iron binding capacity (LIBC), non-protein bound iron (NPBI), ferritin and transferrin were determined at baseline (t=0). After the patients had received anti-emetic therapy, the IV infusion of doxorubicin was started. Immediately after the doxorubicin infusion was completed (t= 0:15 hours) the first sample was obtained for determination of total iron, LIBC and NPBI, followed by the cyclophosphamide infusion. At t=2:30 hours after the doxorubicin administration a second sample (total iron, LIBC and NPBI) was obtained. The following morning (t=24:00 hours) blood was sampled for determination of iron, latent iron binding capacity (LIBC), non-protein bound iron (NPBI), ferritin and transferrin. Baseline and 24 hour measurements were repeated during each subsequent chemotherapy course. At approximately 1 month after chemotherapy blood was sampled for iron, LIBC, non-protein bound iron NPBI, ferritin and transferrin. At approximately 4 months after chemotherapy ferritin and hemoglobin were determined.

LABORATORY PROCEDURES

All assays were performed at the Central Laboratories of Leiden University Medical Center (LUMC).

Ferritin, transferrin, LIBC and total iron

Assays for ferritin, transferrin, LIBC and total iron were performed using routine methodology. Lower limits of detection (inter- and intra-assay variability between brackets) were 0.5 µg/L (<5.35%), 13 mg/L (<1.2%), 4.2 µmol/L (<4.3%) and 0.24 µmol/L (<2.8%) for ferritin, transferrin, LIBC and total iron respectively.

NPBI

NPBI concentrations were measured using a colorimetric method as described previously.(15) Briefly, the serum samples were mixed 9:1 with a 40 mM NTA containing buffer of 5 mM Tris-HCl pH 6.5. After filtration and centrifugation thioglycolic acid sodium salt (3 mM) was added. Measurements were done using a Reader Spectra Max 250 plate reader at 537 nm. The (pooled) sera used for repeated experiments and patient sera were stored at -80 ºC until the measurements (no influence of storage on the NPBI results were found). The lower limit of detection (inter- and intra-assay variability between brackets) was 0.01 µmol/L (<9.2%).

Liver chemistry and hemoglobin

Assays for lactate dehydrogenase (LDH), bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and hemoglobin were measured using routine methodology.

STATISTICAL ANALYSES

To assess the changes within the first course (course 1) measurements at baseline, 0:15, 2:30 (only total iron, LIBC, NPBI) and 24:00 were analyzed (after log-transformation) using a mixed model analysis of variance (SAS proc mixed) with visit (occasion) as repeated factor within subject and time as fixed effects, and subject as random effect. To assess long-term treatment effects the course baseline and follow up measurements of all variables were analyzed the same way. Correction for multiple comparisons was not done because of the exploratory nature of the study. All statistical analyses were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Baseline characteristics

We included 39 patients (23 were scheduled for 4 courses and 16 for 5 courses) with a median age of 49 years (range 30-66 years), mean BMI 25.3 kg/m2 (SD 4.4) and a mean cumulative doxorubicin dose 255 mg/m2 (SD 58) (table 1).

Iron metabolism during the first AC course

At baseline, 32 of the 37 (maximal value: 1.26 µmol/L) obtained samples were positive for NPBI, directly and at 2:30 hours after doxorubicin infusion all samples turned positive for NPBI, ranges were 0.14 - 1.51 µmol/L and 0.09 - 1.45 µmol/L respectively. At 24 hours following the chemotherapy course 26 of the 35 (maximal value: 1.18 µmol/L) obtained samples were positive for NPBI.

Mean NPBI concentration increased (percentage change, 95% confidence interval between brackets) directly after the doxorubicin infusion with 65.7% (23.5 to 122.3%). At 2:30 hours post-dose the increase was 47.3% (14.2 to 90.1%) and at 24 hours no differences were observed (table 2, figure 1).

After a small initial decline, iron increased with 187% (154 to 225%) at 24 hours after the first chemotherapy course. LIBC did not change during the first 2:30 hours post-dose but declined with -75.6% (-82.6 to -65.8%) at 24 hours following chemotherapy. Ferritin was increased with 17.2% (8.3 to 26.9%) at 24 hour after AC administration. Hemoglobin and transferrin did not change significantly during the first course.

Additional analyses showed that the changes (for all parameters) at 24 hours were similar during all subsequent chemotherapy courses.

Long-term effects of chemotherapy on iron metabolism

Ferritin increased by 79.1% (37.1 to 133.9%) during the courses (difference between baseline course 5 and 1) and decreased during the follow up period (table 2, figure 2).

LIBC and transferrin changed minimally over course baseline. NPBI did not change significantly over baseline during the courses.

Hemoglobin level declined during each subsequent chemotherapy course, difference (percentage change, 95%-confidence interval between brackets) between the baseline values of course 5 and 1 was -10.2% (-12.9 to -7.4%). After a full chemotherapy cycle hemoglobin levels increased again.(table 2, figure 2).

Liver function and haemolysis parameters

During the first course bilirubin increased from 6.8 U/L baseline to 9.9 U/L at 24 hours, while LDH declined from 359 U/L to 297 U/L and concentrations of AST and ALT did not change markedly during the courses and concentrations remained within the normal limits.

DISCUSSION

During the first course of doxorubicin in early breast cancer patients an increase in NPBI level occurred almost immediately after doxorubicin infusion and at 24 hours total iron concentration

almost tripled, leading to a complete saturation of transferrin. It was also shown that within 24 hours after administration small rises in serum ferritin were observed and that ferritin increased gradually over baseline during the AC courses.

An intriguing finding of this study was that almost directly after DOX infusion NPBI increases, indicating that even relatively low doses of DOX as employed in the adjuvant setting are potentially harmful. The observation that the peaks are lower compared to previous studies could relate to the fact that we used an optimized spectrophotometric method, which allowed us to determine free iron concentrations more realistically.(15) However, it cannot be ruled out that the increase in NPBI is dose-dependent and that the dose of doxorubicin that we studied did not provoke a similar iron overload as in patients receiving high dose chemotherapy. The fact that in other studies NPBI was detectable for a longer period of time also supports this hypothesis.(8-10) The current opinion that NPBI is not detectable in (healthy) subjects without apparent iron overload can be questioned based on the data in this study, as we found NPBI levels prior to chemotherapy in a majority of patients. This is in keeping with other data indicating that under physiological conditions NPBI can be present, although it is important to note that in these papers different assays were used.(8;15) Obviously, our population consisted of patients and it cannot be excluded that the presence of NPBI before dosing with anthracyclines reflects the disease state, but we consider this unlikely in view of the fact that they were treated curatively and did not have macroscopic residual tumor. Thus, it seems that also under conditions without apparent iron overload circulating NPBI can be present, although its role is unclear.

The observation that iron concentrations increase shortly after chemotherapy in patients who are tumor free may help to further understand the possible source of iron that is not immediately apparent from previous experiments. Previous experiments have suggested that the possible sources of iron includes destructed tumor cells, impaired erythropoiesis, damage to the gastrointestinal mucosa, hemolysis and liver injury. (6;8-11;16-18)However, in our surgically tumor free population, tumor lysis, hepatic injury, and also hemolysis does not seem a major contributor for the observed iron bursts, as tumor mass was negligible, and bilirubin, transaminases and LDH did not change considerably during the courses. Although erythropoiesis is affected by chemotherapy, this does not seem to be the cause of the iron peaks as these occurred almost immediately after AC administration, while the effects on erythrocytes occurred later and no signs of hemolysis were present. We hypothesize that AC-chemotherapy provoke diffuse (low-grade) injury to several tissues, such as the gastro-intestinal mucosa, splenic cells, etc., which together cause sufficient cellular damage to provoke the observed iron peaks.

We also found small increases of ferritin within 24 hours after each course which seems to be cumulative and results in a gradual increase over the entire treatment period. The iron-storage protein ferritin, which is abundantly present intracellularly and in small amounts in serum, is associated with total body iron-store in healthy individuals. Elevated ferritin levels have been reported to result from increased synthesis in response to inflammation and because of cellular damage and/or a nonspecific response of the reticuloendothelial system to an increased tumor load in cancer patients.(19-24) Also, it has been suggested that increased ferritin levels could protect against tumor proliferation.(21) However, a response to tumor proliferation is unlikely in our population of early stage breast cancer patients. We cannot exclude that the rise is caused by an acute phase response to a (low-grade) inflammatory response to the administered chemotherapy, but the absence of increases in hsCRP (results not shown) render this explanation less likely. Another possibility is that (at least) some of the observed changes reflect a direct effect of doxorubicin on iron metabolism or a protective response to the chemotherapy-induced iron overload. This explanation would be in keeping with the notion that pre-clinical studies have shown that doxorubicin has marked effects on intra-cellular iron homeostasis by increasing accumulation of iron in ferritin, inducing increased expression of ferritin and inhibiting release of iron from ferritin.(4)

A potential drawback of our study is that we attribute the changes to effects of AC-chemotherapy as we (for obvious reasons) did not include a placebo control. However, we consider it unlikely that the changes that we observed can be attributed to spontaneous time (circadian) effects. Also, it is unlikely that

cyclophosphamide contributed greatly to the increased release of NPBI, as the maximal increase in NPBI was already present immediately after completion of the doxorubicin infusion and before the administration of cyclophosphamide.

In summary, we found that a single IV dose of doxorubicin immediately results in occurrence of highly toxic NPBI in the circulation. This could help to further understand the *in vivo* mechanism of doxorubicin toxicity and may produce leads into protective agents for this.

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Table 1 Baseline characteristics

	Female breast cancer patients (n = 39)	
	MEAN	SD
Iron parameters		
Hemoglobin - mmol/L	7.7	0.7
Total iron - µmol/L	16.5	6.7
NPBI - µmol/L	0.44	0.26
LIBC - µmol/L	43.9	13.6
Ferritin - µg/L	70.7	62.7
Transferrin - g/L	2.61	0.60
Liver chemistry		
ALT - U/L	24.4	24.9
AST - U/L	31.7	16.5
LDH - U/L	393	222
Bilirubin - mg/dL	7.8	4.5

Table 2 Percentage change from course baseline (95% confidence interval) for total iron, latent iron binding capacity (LIBC), non-protein bound iron (NPBI), hemoglobin haemoglobin, ferritin and transferrin during the first chemotherapy course

Time post-dose	**0:15 HR**	**2:30 HR**	**24 HR**
Total iron	-15.4 (-19.8 TO -10.8)	-25.1 (-33.0 TO -16.2)	187.1 (153.7 TO 225.0)
LIBC	-2.1 (-6.8 TO 2.8)	3.1 (-5.0 TO 11.9)	-75.6 (-82.6 TO -65.8)
NPBI	65.7 (23.5 TO 122.3)	47.3 (14.2 TO 90.1)	-10.6 (-44.7 TO 44.5)
Ferritin			17.2 (8.3 TO 26.9)
Transferrin			6.0 (-8.9 TO 23.3)
Hemoglobin			-2.4 (-4.2 TO -0.5)

Table 3 Percentage change from baseline of course 1 (95% confidence interval) for hemoglobin, ferritin and transferring for each chemotherapy course and at follow up.

	Hemoglobin	**Ferritin**	**Transferrin**
Course 2	-4.4% (-6.6 TO -2.1)	19.9 (-1.2 TO 45.4)	-0.8 (-15.1 TO 15.9)
Course 3	-7.8 (-10.5 TO -4.9)	46.9 (14.4 TO 88.7)	-3.8 (-18.2 TO 13.2)
Course 4	-8.3 (-10.5 TO -6.0)	59.3 (29.2 TO 96.3)	2.0 -13.3 TO 19.9)
Course 5	-10.2 (-12.9 TO -7.4)	79.1 (37.1 TO 133.9)	0.2 (-14.7 TO 17.8)
Follow up 1 month	-4.4 (-6.7 TO -2.0)	68.6 (37.9 TO 106.1)	14.9 (-2.0 TO 34.8)
Follow up 4 months	6.6 (4.1 TO 9.1)	25.0 (2.6 TO 52.2)	N/A

Figure 1 Mean (standard deviation as error bars) serum concentrations of non-protein bound iron (a), total iron (b) and latent iron binding capacity (c) during the first course. *significant p < 0.001

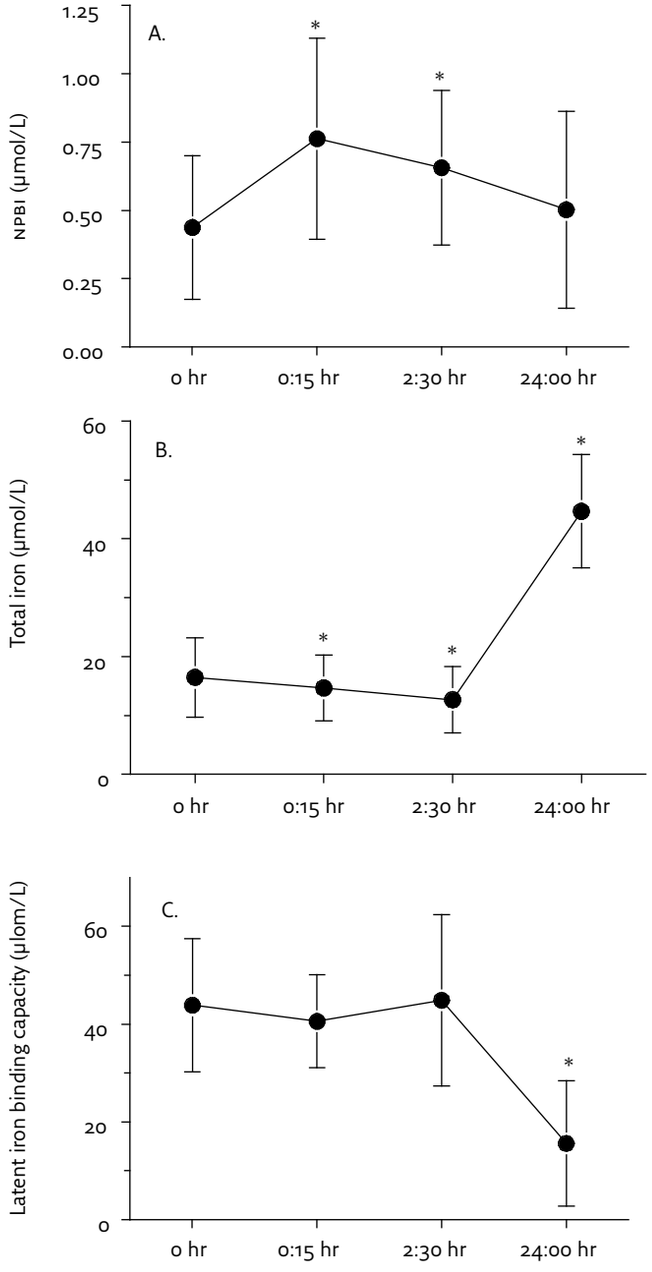

Figure 2 Mean (standard deviation as error bars) serum concentration of hemoglobin (a), transferrin (b) and ferritin (c) at baseline and 24 hours after each chemotherapy course and during the follow-up period.