On the pro-oxidant effects of haemozoin

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Abstract Haemozoin (Hz) is a haem aggregate produced in some blood-feeding organisms. There is a general belief that Hz formation would be a protective mechanism against haem toxicity. Here we show that when aggregated into Hz, haem is less deleterious than its free form. When haem was added to phosphatidylcholine (PC) liposomes, there was an intense stimulation of oxygen consumption, which did not occur when Hz was incubated with the same preparation. Evaluation of oxygen radical attack to lipids, by measurement of thiobarbituric acid reactive substances (TBARS), showed significantly lower levels of lipid peroxidation in samples containing PC liposomes incubated with Hz than with haem. However, TBARS production induced by Hz was much higher when using 2-deoxyribose (2-DR) as substrate, than with PC liposomes. Spin-trapping analysis by electron paramagnetic resonance (EPR) of Hz and *tert*-butylhydroperoxide (*tert*-BuOOH) showed that production of methoxyl and *tert*-butoxyl radicals was only slightly reduced compared to what was observed with haem. Interestingly, when large Hz crystals were used in 2-DR TBARS assays and *tert*-BuOOH EPR experiments, the pro-oxidant effects of Hz were strongly reduced. Moreover, increasing concentrations of Hz did not induce erythrocyte lysis, as occurred with haem. Thus, the reduced capacity of Hz to impose radical damage seems to result from steric hindrance of substrates to access the aggregated haem, that becomes less available to participate in redox reactions.

Key words: Haemozoin; Haem; Free radical; Oxidative stress

1. Introduction

Haem constitutes an important molecule in many biological reactions, including oxygen transport, respiration, photosynthesis and drug detoxification. However, free haem is a powerful generator of reactive oxygen species and can damage a variety of biomolecules [1]. Haem can also associate with phospholipid membranes, altering the bilayer structure which leads to cell disruption [2,3]. Parasitic organisms that have vertebrate blood as their sole food source, therefore face a special situation and, in the course of their evolutive history, had to develop adaptations to avoid the deleterious effects of high concentrations of free haem found in their food. Haem detoxification can be accomplished by its enzymatic degradation by haem oxygenase [4] or non-enzymatically by reduced glutathione [5]. Furthermore, proteins such as the *Rhodnius* haem-binding protein, from the hemolymph of a blood-sucking insect [6,7] are capable to interact with haem and form complexes that do not promote free radical generation. In malaria parasites, it has been demonstrated that free haem is sequestered inside the digestive vacuole into a dark brown crystalline aggregate called malaria pigment or haemozoin (Hz) [8,9]. This pigment has recently been found in other blood-feeding organisms such as *Rhodnius prolixus*, an insect [10,11], in the helminth *Schistosoma mansoni* [12] and in the parasitic protozoan *Haemoproteus columbae* [13].

It is generally accepted that Hz would be a less toxic disposable form of the haemoglobin-derived haem. However, data from the literature showed that when ingested by monocytes or macrophages, Hz is capable to affect various cellular functions such as oxidative burst, phagocytosis and antigen processing [14–18]. In fact, ingestion of Hz by human monocytes results in formation of lipoperoxides and toxic hydroxyaldehydes such as 4-hydroxynonenal (HNE) [19]. However, in a recent work, Omodeo-Salè and co-workers demonstrated that pro-oxidant effects of β-haematin were dependent of the oxidation status of the membranes, as the levels of pre-existing hydroperoxides would be essential for free radicals generation and lipid peroxidation induced by this haem aggregate [20]. Therefore, this apparent controversy and the scarcity of information about the reactivity of the haem after aggregation into Hz, led us to further investigate this issue. In this report we show, by different methods, that Hz exhibits less deleterious effects than free haem and that this is not due

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to a reduction in haem reactivity, but is dependent of Hz crystals' size.

2. Materials and methods

2.1. Reagents

Thiobarbituric acid (TBA), haemin, 2-deoxyribose (2-DR), deferoxamine mesylate, phosphatidylcholine (PC; Azolectin), DMPO, *tert*-butylhydroperoxide (*tert*-BuOOH) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Animals and Hz extraction

R. prolixus were maintained at 28°C and 80% relative humidity. Hz was extracted from the midgut of adult females as described earlier [11]. Samples were centrifuged at $10\,000\times g$ (Sorvall RC2-B) for 10 min and the pellet was resuspended in 0.1 M bicarbonate buffer, pH 9.1, and 2.5% SDS. Samples were centrifuged at $13\,000\times g$ in a microcentrifuge (Eppendorf 5417R) for 10 min. This procedure was repeated twice and after that the pellet was washed twice with water. Hz quantification was carried out by adding 1 ml of 0.1 M NaOH, vortexing the samples for 30 min, followed by determination of haem content at 400 nm in an Ultrospec U2000 Pharmacia spectrophotometer. Non-sonicated Hz (NSHz) was prepared by resuspension of dried Hz crystals in water and shaking for 1 min. Sonicated Hz (Hz) was prepared by ultrasound microdispersion in a probe sonifier (Branson sonifier 250) with an output power of 200 W for 5 s, just before the experiments. Sonication conditions were: duty cycle, 90% constant; output control, 3. De-aggregated Hz (dHz) was prepared by incubating extracted Hz in 0.1 M NaOH and vortexing for 10 min prior the experiments. In this case, after de-aggregation by NaOH, samples were neutralised with sodium phosphate buffer. For measurements of Hz crystals' size, three adult females of *R. prolixus* were dissected to obtain midgut luminal content. Midguts were poured into a tube with deionised water, gently shaken for 10 s and left in the bench for 10 min. After that, the supernatant was removed and an aliquot of this sample was used in the measurements.

2.3. Field emission scanning electron microscopy (FESEM) and morphometry of Hz crystals

For observations of Hz crystals, pigments were dropped over a stub support and kept at 37°C until dryness. After dry, samples were coated with carbon and directly observed in a JEOL JSM-6340F field emission scanning electron microscope at an accelerating voltage of 5.0 kV. Four different fields of each sample were acquired and the size of crystals was determined by manual measurements with a photo editing software (Adobe© Photoshop 5.0).

2.4. PC liposomes preparation

PC liposomes were prepared by dissolving 12 mg of Azolectin in 1 ml of 10 mM HEPES buffer, pH 7.4. This suspension was shaken for 1 min and subsequently sonicated for 10 min. This procedure was repeated until a homogeneous suspension was obtained.

2.5. Oxygen consumption

Lipid peroxidation of PC liposomes was monitored by measuring O_2 consumption using a Clark-type oxygen electrode (YSI, Model 5775, Yellow Springs, OH, USA) calibrated to 100% with air-saturated buffer at room temperature. Cuvettes contained 1.5 ml of 50 mM sodium phosphate buffer, pH 7.4, 17 μM deferoxamine and 0.5% of PC liposomes. Subsequent additions are indicated in figure legends. Hz used in this experiment was previously sonicated as described in Section 2.2.

2.6. Thiobarbituric acid reactive substances (TBARS) assay

Lipids were oxidised by adding 15 μM of haem, NSHz, Hz or dHz to 0.5% PC liposomes. These reaction mixtures were incubated at 37°C for 1 h and then were added 200 μl of 1% TBA in 0.05 M NaOH and 200 μl of acetic acid. Tubes were heated at 95°C for 15 min, cooled, and the absorbance determined at 532 nm. When 2-DR was used as a substrate for oxidation, the reaction mixture (100 μl) contained 2.8 mM 2-DR, 20 mM potassium phosphate buffer, pH 7.4, and 80 μM deferoxamine. Reactions were started by adding 15 μM of haem, NSHz, Hz, or dHz. After 1 h at 37°C, 200 μl of 1% TBA in 0.05 M NaOH was added followed by 200 μl of acetic acid. Tubes were heated at 95°C for 30 min, cooled, and the absorbance was determined at 532 nm.

2.7. Electron paramagnetic resonance (EPR) spin-trapping

Methoxyl and *tert*-butoxyl radical generation induced by haem or by Hz was evaluated by EPR spectroscopy coupled to spin-trapping analysis. Samples contained 10 μM haem, NSHz, Hz, or dHz, 38 mM *tert*-BuOOH and 40 mM DMPO. EPR spectra were obtained using a Bruker-ER 380 spectrometer (Bruker, Germany) operating at a microwave power of 6.5 mW with a 100 kHz modulation frequency. After mixing all the reagents, the samples were transferred to a quartz flat cell and spectra were acquired within 15 s. The instrument settings were: scan time, 42 s; scan width, 63 G; time constant, 41 ms; and modulation width, 1.0 G. DMPO was diluted 1:2 in water and purified by treatment with activated charcoal [21]. All solutions were prepared in Milli-Q water and were made freshly.

2.8. Hemolysis

Rabbit erythrocytes, 0.5% v/v, were incubated for 2 h at 37°C in 0.15 M sodium phosphate buffer, pH 7.4, with different concentrations of haem, and Hz as stated in the figure legends [2]. After incubation, tubes were centrifuged at $1000\times g$ for 2 min and the amount of haemoglobin in the supernatant was determined spectrophotometrically at 540 nm. Hz used in this experiment was previously sonicated as described in Section 2.2.

3. Results

3.1. Hz crystals' size determination

The extraction procedures of Hz from *R. prolixus* midgut

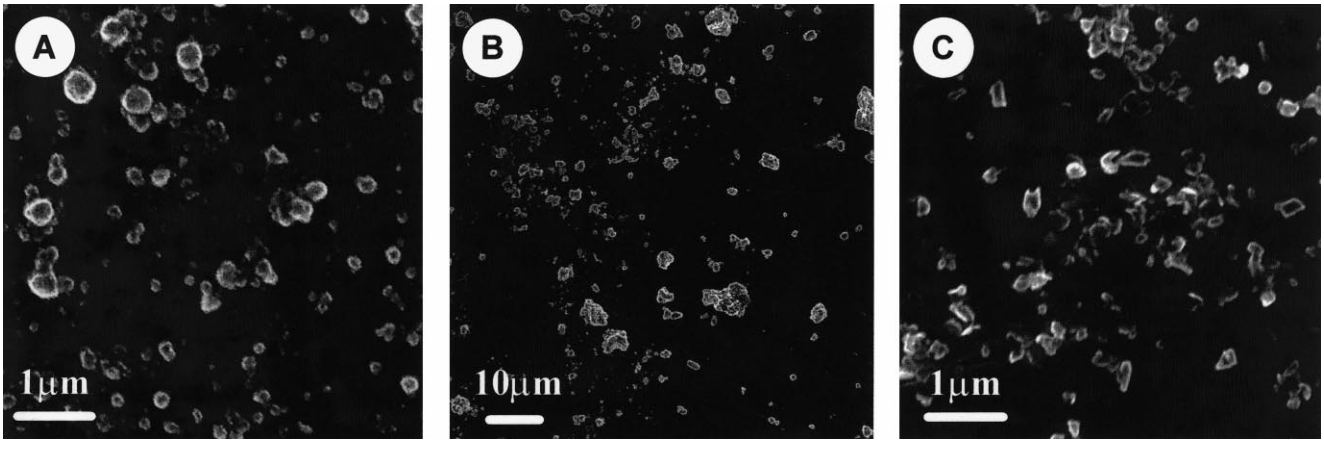
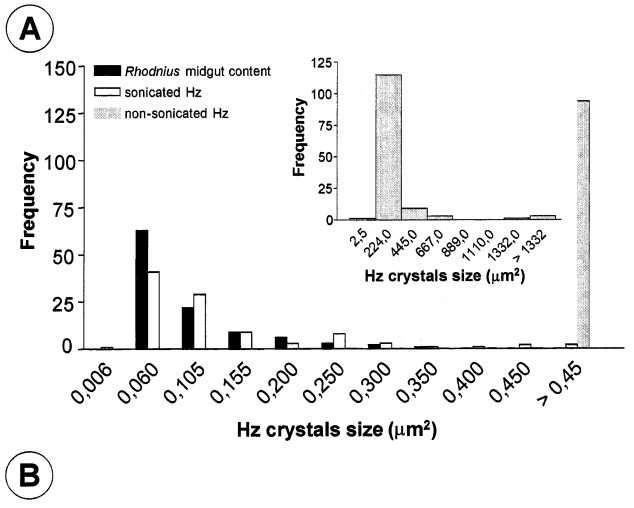


Fig. 1. FESEM of Hz crystals. RMC (A), NSHz (B) and Hz (C) crystals were observed with a JEOL JSM-6340F field emission scanning electron microscope.

usually produce very large particles, which can be seen by bare eyes. However, personal observations and previous analysis of *R. prolixus* midgut luminal content [10,11] suggest that the crystals found inside the midgut were much smaller than those obtained after Hz extraction methods. Since this difference in crystals' size could introduce an artifact in our measurements of pro-oxidant activity of Hz, we tried to produce particles of size similar to those found in *R. prolixus* midgut. Hz particles were observed by FESEM, and shown in Fig. 1. *Rhodnius* midgut content (RMC; Fig. 1A) revealed particles of different sizes but not larger than 1 μm. The observation of Hz granules (NSHz; Fig. 1B) obtained after subjecting RMC samples to Hz extraction procedures, revealed very large Hz particles. However, when NSHz samples were microdispersed for 5 s by ultrasound and observed by FESEM (Hz; Fig. 1C), we noted that the size of these particles were comparable to those found inside *Rhodnius* midgut. Fig. 2 shows the measurements of RMC, NSHz and Hz crystals' size observed in Fig. 1. Measurements of RMC samples revealed that crystals from 0.0063 μm^2 up to 0.0558 μm^2 were the most frequently observed (Fig. 2A, black bars) and the average size of particles was 0.069 μm^2 (Fig. 2B), while in NSHz, crystals from 2.4 μm^2 to 223.9 μm^2 (Fig. 2A, inset) were predominant and the mean size of these granules was 123.4 μm^2 (Fig. 2B). However, particles from 0.0063 μm^2 to 0.0558 μm^2 were most frequent when extracted Hz crystals were submitted to sonication just before the measurements (Fig. 2A, open bars) and had an average size of 0.086 μm^2 (Fig. 2B). Therefore, sonication of Hz extracted from *R. prolixus* midgut produced particles that were indistinguishable in size to those intact crystals found in vivo. For this reason, in the following experiments we compared the pro-oxidant activity of Hz in three different aggregation states (NSHz, Hz and dHz particles).



	Mean size (μm^2)	s.e.m.	n
***Rhodnius* midgut**	0.069	0.006	106
Non-sonicated Hz	123.439	30.999	132
Sonicated Hz	0.086	0.006	101

Fig. 2. Morphometry of Hz crystals. A: Frequency distribution of Hz crystals' size from *Rhodnius* midgut (black bars), Hz (open bars) and NSHz (grey bars). The inset shows a more detailed frequency distribution of data from NSHz. B: Determination of mean ± S.E.M. of Hz particles' size. Measurements were made with Adobe© Photoshop 5.0 software.

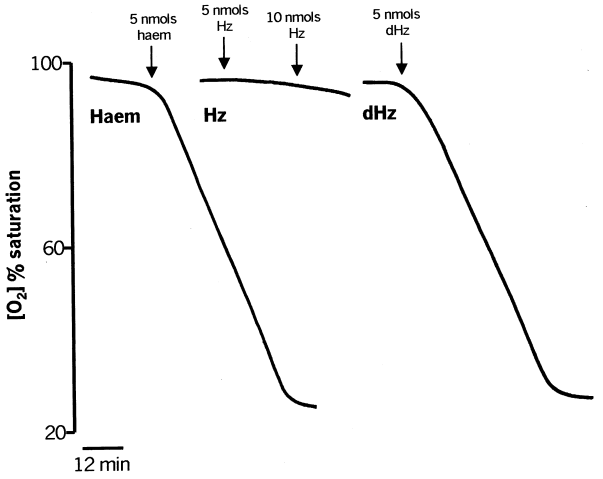


Fig. 3. O_2 consumption induced by haem and Hz. Lipid peroxidation was monitored with a Clark-type electrode at room temperature. Haem: Addition of 5 nmol of haem; Hz: two subsequent additions of 5 nmol of Hz and a further addition of 10 nmol. Addition of haem and Hz are shown in the figure. dHz: Addition of 5 nmol of Hz previously de-aggregated by NaOH.

3.2. Oxygen uptake

Molecular oxygen participates in the propagation phase of lipid peroxidation reaction chain and therefore oxygen consumption is an index of radical damage to lipids. The oxygen uptake during oxidation of PC liposomes was mea-

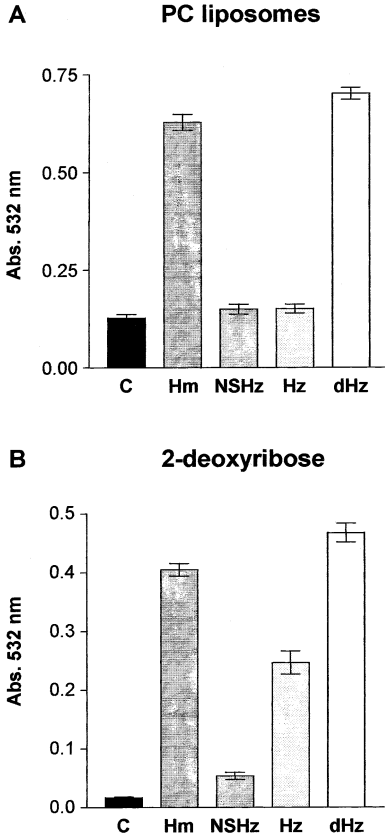


Fig. 4. Lipid and carbohydrate oxidation by haem and Hz. TBARS production induced by 15 μM of haem or Hz in different aggregation states using (A) PC liposomes or (B) 2-DR as substrate for oxidation. Hm, Haem. C: Control samples without addition. Data are presented as mean ± S.D. for four determinations.

sured with a Clark oxygen electrode in order to compare the pro-oxidant activity of Hz and haem (Fig. 3). Although a rapid decrease in O_2 concentration was observed when haem was added to the cuvette, no oxygen consumption was induced by Hz. O_2 consumption identical to that produced by haem was obtained when 5 nmol of dHz was added to the system.

3.3. TBARS assay for phospholipid and carbohydrate oxidation

Incubation of extracted Hz from *R. prolixus* midgut with PC liposomes produced very low levels of TBARS compared to haem (Fig. 4A). Similar results were obtained when linolenic acid micelles were used as substrates for oxidation (data not shown). In all the experiments using lipidic substrates, the size of Hz crystals did not affect TBARS formation. In contrast, when 2-DR, which is a low molecular mass hydrophilic substrate, was used for oxidation, a significant amount of TBARS was formed after incubation with Hz (Fig. 4B). Interestingly, when NSHz crystals were used, carbohydrate oxidation occurred in a expressively lesser extent than in Hz samples. Disruption of Hz by NaOH (dHz), resulted in TBARS levels comparable to haem, both for PC liposomes and 2-DR.

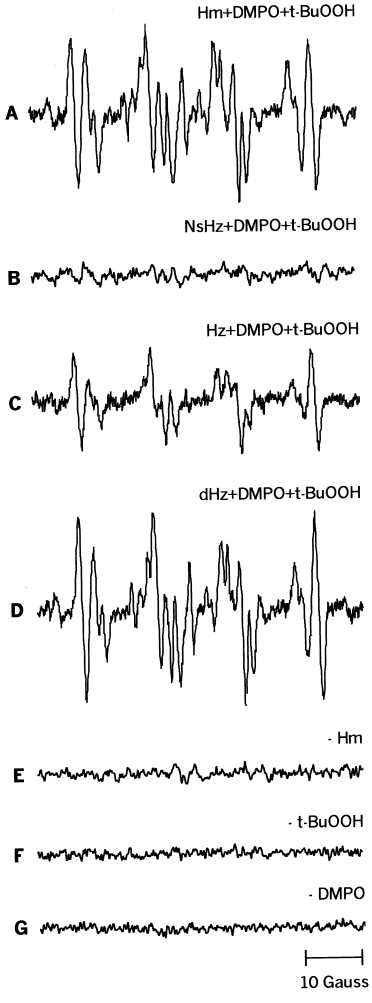


Fig. 5. EPR spin-trapping analysis of free radicals produced by incubation of haem or Hz with *tert*-BuOOH and DMPO. Samples contained 10 μM haem, Hz or dHz, 38 mM *tert*-BuOOH and 40 mM DMPO. A: Spectrum of haem. B: As (A), but with NSHz instead of haem. C: As (A), but with Hz instead of haem. D: As (A), but with dHz instead of haem. E: As (A), but without haem. F: As (A), but without *tert*-BuOOH. G: As (A), but without DMPO. EPR spectra were obtained using a Bruker-ER 380 spectrometer operating at a microwave power of 6.5 mW with a 100 kHz modulation frequency, scan time 42 s, scan width 63 G, time constant 41 ms, modulation width 1.0 G.

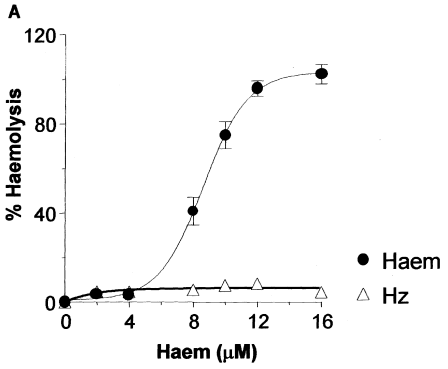


Fig. 6. Haem aggregation into Hz reduces haem-induced haemolysis. Aliquots of 0.5% suspension of rabbit erythrocytes were incubated for 2 h at 37°C with increasing concentrations of haem (●) or Hz (△). Data are presented as percentage of haemolysis compared to erythrocytes incubated with distilled water. Bars represent mean ± S.D. for four determinations.

3.4. Free radicals detection by EPR spin trapping

In order to evaluate the reactivity of the haem molecules present in the Hz crystals we compared the generation of methoxyl and *tert*-butoxyl radicals by haem and Hz by means of EPR spectroscopy coupled to spin-trapping analysis. Incubation of *tert*-BuOOH with haem in the presence of DMPO resulted in a complex spectrum comprising adducts derived from *tert*-BuOOH decomposition and DMPO (Fig. 5). The hyperfine coupling constants of these adducts indicate that methoxyl ($a^N = 14.08$ G, $a^H_\beta = 10.7$ G and $a^H_\gamma = 1.3$ G) and *tert*-butoxyl ($a^N = 14.8$ G and $a^H_\beta = 16.95$ G) were the predominant radical species generated. This result was in agreement with data reported for reactions of hydroperoxides with haematin and haem-proteins [22–24]. The same concentration of Hz produced radical adducts, but to a lesser extent than haem. Interestingly, NSHz crystals led to the detection of very low methoxyl and *tert*-butoxyl signals. Hz disruption by previous NaOH treatment produced a spectrum identical to that of haem with similar signal intensity. No radical formation was observed on the absence of haem, DMPO or *tert*-BuOOH.

3.5. Erythrocyte lysis

Heme can associate with phospholipid bilayers, destabilising membrane structure and leading to cell lysis [2,3]. Therefore, we used erythrocyte lysis as a model system to evaluate Hz capacity to induce cell disruption. A dose–response curve of haemolysis was observed when erythrocytes were incubated with increasing concentrations of haem (Fig. 6). In contrast, haemolysis was not observed when cells were incubated with increasing amounts of Hz. Moreover, it did not occur when cells were incubated with β-haematin, which is a haem aggregate identical to Hz, produced under non-physiological conditions (data not shown).

4. Discussion

In the present work we demonstrated that aggregation of the haemoglobin-derived haem into Hz reduces its deleterious effects. Thus, haem aggregation seems to have a pivotal role in order to reduce the pro-oxidant effects of haem after release from globin chain by forming a less harmful disposable by-product.

Haemoglobin digestion, haem release and Hz formation take place inside the midgut of *R. prolixus* and *S. mansoni* or in *Plasmodium* food vacuoles, where intense hydrogen peroxide production has been reported [25,26]. Since haem is able to decompose peroxides with concomitant degradation of the porphyrin ring, as observed by disappearance of the Soret band [27], aggregation into Hz would be an efficient way to avoid haem destruction and iron release. In fact, a previous report showed that β-haematin crystals incubated with hydrogen peroxide did not change the Soret band as occurred with haem, indicating that haem molecules in β-haematin are not as reactive towards peroxides as they are in the free form [28].

Previous works showed that Hz has been involved in many biological activities such as impairment of phagocytic function [16], induction of lipoperoxidation [18,19], HNE and hydroxyeicosatetraenoic acids production [29,30]. Moreover, inhibition of macrophage accessory cell activity was observed in Hz-loaded macrophages, which would possibly occur due to the uptake and accumulation of Hz in their cytoplasms [31]. Later, further research indicated that Hz has also been involved in immunomodulation of macrophage functions affecting antigen processing, chemokine and cytokine secretion [16,17,32–36]. However, in the present work we provide evidence that Hz exert less deleterious effects than free haem, by means of evaluating three different parameters: cell damage, molecular damage and direct observation of free radical generation. The apparent controversy between our data and those reports can be explained assuming that free iron, and not Hz, would be responsible for lipid peroxidation observed in Hz-loaded monocytes [16]. Malaria parasites digest up to 80% of host cells' haemoglobin, and this process generates a complex mixture consisting in haemoglobin peptides, amino acids, haem, Hz and free iron inside the food vacuoles [37], and iron is known to be released from Hz in vitro in conditions quite similar to those found in the macrophage phagolysosome [16]. The results presented here are in agreement with the hypothesis that Hz is a less toxic form of haem, since the possibility of free iron-induced radical generation was excluded in our experiments, due to the presence of the iron-chelator deferoxamine.

Although it is not well established whether haem can really act as a Fenton reagent, generating hydroxyl radical, several studies indicated that when haem degradation is observed, hydroxyl radicals can be detected [38]. Moreover, the inhibition of haem degradation and iron release by enzymatically generated H_2O_2 occurred after incubation with hydroxyl radical scavengers, reducing agents or antioxidant enzymes, such as superoxide dismutase and catalase [39]. Oxidation of 2-DR has been attributed to the highly reactive hydroxyl radical [40,41]. However, attempts to observe hydroxyl radical formation upon incubation of haem with hydrogen peroxide and DMPO by EPR led only to the detection of oxidised species of DMPO (data not shown). Whatever the mechanism, our results show that there was an extensive oxidation of 2-DR when haem, Hz and dHz, but not NSHz, were used as pro-oxidant reagents.

NSHz batches from *R. prolixus* midgut were obtained as large crystals with 123 μm^2 of average size (which does not occur in vivo). We observed that the pro-oxidant activity of Hz depended on the crystals' size. Reduction in Hz particle size led to high levels of oxidation of 2-DR or *tert*-BuOOH similar to those found when the same substrates were incubated with free haem, as showed in Figs. 4B and 5. Nevertheless, when lipidic micellar substrates were used (PC and linolenic acid), even Hz crystals showed very low pro-oxidant capacity. This could be explained by assuming that only the most superficial molecules of haem in Hz would be available to participate in radical formation. Therefore, the contact area of the crystal available for water-soluble substrates is certainly much higher than that available to interact with large supramolecular structures such as lipid micelles. An increase of surface contact of the haem molecules with its surrounding environment is expected to occur when Hz crystals are submitted to sonication. This hypothesis may also explain why incubation of Hz in the presence of hydrogen peroxide at pH 5.5, simulating the monocyte phagolysosome environment, results in the release of only a minor fraction of the total iron content, suggesting that only superficial iron is removed [16]. In contrast, a recent report concluded that differences in susceptibility of macrophages cell lines to oxidative damage induced by β-haematin could not be attributed to the release of iron from protoporphyrin molecules [42]. Notwithstanding, in a cellular system it is conceivable that hydrophilic, low molecular mass substrates present in macrophage phagolysosomes could be oxidised by β-haematin, accounting for a TBARS production similar to haem. Moreover, it is important to notice that β-haematin batches used in their experiments were previously microdispersed by sonication (a condition which increases its pro-oxidant effects) [42]. In fact, Hz crystals had a reduced size and consequently an increase in molecules of haem available for oxy-reduction reactions at the crystals' surface and this could account for their higher oxidative capacity. In support to this view, a recent work describes a reduced pro-oxidant activity of β-haematin when incubated with lipidic substrates [20]. This work demonstrates that the pro-oxidant activity of β-haematin is dependent of the amount of hydroperoxides in their samples. Based on these findings, it is conceivable that in the PC liposomes, used in our experiments, small amounts of hydroperoxides could explain why our lipid substrates were not so damaged by Hz as reported for arachidonic acid and large unilamellar vesicles in Omodeo-Salè and colleagues' work. Interestingly, studies involving lipid peroxidation induced by ferritin and haemosiderin indicates that production of haemosiderin in iron-overload conditions such as thalassemia represents a biologically relevant protection since it decreases the ability of iron to promote free radical generation [43–45]. Formation of TBARS induced by haemosiderin in vitro indicates that only about 10% of total iron in haemosiderin is involved in lipid peroxidation [43]. This suggests that, similar to our observation with Hz particles, only the superficial iron in haemosiderin would be able to generate free radicals.

The catalytic decomposition of hydroperoxides by haem–iron complexes results in free radicals generation and it is believed to induce lipid peroxidation and protein damage [22,23]. Incubation of haem with *tert*-BuOOH in the presence

of the spin-trap DMPO led to the detection of methoxyl and a *tert*-butoxyl radical adducts as previously observed [22–24], while lower levels of radical adducts were produced with Hz (Fig. 5). Our results are in agreement with a recent work, which indicated that previously reported peroxyl radicals, generated by incubation of chloroperoxidase, haem or haem-proteins with *tert*-BuOOH, are likely to be methoxyl and *tert*-butoxyl radicals adducts rather than methylperoxyl [24]. The authenticity of these adducts was reinforced since the presence of 500 U of superoxide dismutase did not change the spectra (data not shown). In order to exclude the presence of free iron in samples of haem or Hz used for EPR assays, we incubated these compounds with deferoxamine and DMPO based on a previous report which showed that deferoxamine enhances Fe^{+2} oxidation by molecular oxygen, producing hydroxyl radical [46]. No hydroxyl radical adducts were observed when haem or Hz, DMPO and deferoxamine were incubated, therefore excluding the presence of free iron in our measurements (data not shown).

Although it has been shown that malarial pigment could exert toxic effects in both cellular systems and in vitro [14–20,29–36], our report indicates that haem aggregation into Hz does not reduce haem intrinsic reactivity. The dramatic reduction in the generation of oxidising species upon Hz formation seems to result from steric hindrance of the inner core of the Hz crystal, which would be less available for participating of oxy-reduction reactions. Therefore, Hz formation in the blood-feeding organisms [8,10–13] seems to be an important mechanism to prevent generation of free radicals by haem released upon haemoglobin digestion.

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