Author's response to reviews

Title: Homeostatic response under carcinogen withdrawal. Heme oxygenase 1 expression and cell cycle association

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Author's response to reviews: see over
Dear Editor:

Please note the attached revised manuscript entitled: “HOMEOSTATIC RESPONSE UNDER CARCINOGEN WITHDRAWAL. HEME OXYGENASE 1 EXPRESSION AND CELL CYCLE ASSOCIATION”. We answered to all the comments addressed by the peer reviewers and made the formatting changes requested.

Formatting changes requested:
- Authors’ competing interests were included in page 9.
- Authors’ contributions were included in page 10.

Here we give a point by point list with all the answers to the referees’ concerns:

**Referee 1: Inge Bauer**

**Major Revisions:**

1) Even though in this work we didn’t assess the oxidative status, previous studies reported by us (included as a reference in the manuscript), clearly demonstrated that after 35 days of DAB feeding, oxidative stress signals were triggered which were maintained for longer intoxication protocols and HO-1 expression was highly induced (Gerez et al, 1997; Vazquez et al, 2002; Sacca et al, 2004). Therefore, we can assume that animals after 78 & 89 days were under this same oxidative status and that the HO-1 decrease after DAB dietary deprivation correlates with the amelioration of this condition.

2) Referred to the group size, n=6 in all groups. There are three groups: control (n=6), HC (n=6) & HR (n=6) Total n=18. The size number was clarified in Materials & Methods (page 4). Figure 2&3 legends were corrected (page 13), it was a typing mistake. The pattern of protein expressions was consistent for all the animals (n=6). Nevertheless we showed only one WB corresponding to the most representative result.

3) In Figure 4 labels (a-f), were modified and control, HR and HC identifications were added. The pictures were re-localized to make it clearer.

In the text the two last paragraphs in page 7 were modified as follow:

The HC group showed: necrotic tissue foci with mild (+/-) or absence (-) of HO-1 immunoreaction surrounded by regenerative and normal HO-1 positive (++/++++) hepatocytes (Fig.4a upper-left angle) and hepatic macrophages hyperplasia with increased HO-1 immunoreaction (+++) (Fig.4b); morphological and immunohistochemical “altered hepatic foci lesions” (AHF) with central disposed large irregular hepatocytes with decreased (-/+ HO-1 expression and intense (+++) immunostaining periphery hepatic macrophages (Fig. 4d). The HR group showed: regeneration tissue foci with few intense (+++) immunostaining hypertrophic rounded cells (Fig.4e) and the remaining hepatocytes with HO-1
expression similar (+++) to the normal control group (Fig. 4c), and absence of
tissue necrosis features with normal (+++) HO-1 expression (Fig. 4f).

Legend Figure 4 (page 13) was modified as follows:

Immunohistochemical results: HC group: a) non-apoptotic necrotic tissue foci
(white broad arrow), regenerative and normal HO-1 positive hepatocytes
(upper-left angle, black arrows) and b) hepatic macrophages hyperplasia (black
arrows heads ) and altered hepatocytes (black arrows); d) “altered hepatic foci
lesions (AHF)” with decreased HO-1 expression central hepatocytes (black
arrows) and surrounded by macrophages (black arrows heads )

HR group: e) regenerative tissue foci with few intense staining rounded cells
(white broad arrows), hepatocytes (black arrows ) and few macrophages (black
arrows heads ) with similar normal tissue HO1 expression; f) normal HO-1
expression and absence of tissue necrosis features in HR tissue hepatocytes
(black arrows ) and macrophages (black arrows heads). Control group: c)
normal HO1 hepatic tissue (black arrows) and macrophages (black arrows
heads) expression. (Final Magnification x 312.5).

The terms mild, moderate or intense for the assessment of immunostaining are
commonly used and they refer to the intensity of the staining.

Regarding Figure 4a legend “and normal HO-1 positive hepatocytes…”, “normal”
means a morphological cell resemblance to the histological normal hepatocyte in
comparison with morphological histological abnormal hepatocyte such as dysplastic,
hyperplastic and atypical ones. Due to the technique employed, the ontogenic
hepatocyte evolution alterations or the functional abnormalities can’t be used as
diagnostic criteria, so morphological characteristics were the only diagnostic criteria
used.

Regarding Figure 4b and d: final magnification x312.5 in all cases (This was added
in Figure legend 4, page 13)

Regarding Figure 4b: The macrophages, one of the sinusoidal lining cells, were
identified as resident macrophages (so-called Kupffer cells) by morphology and
conventional histological stains (H&E and PAS) in histological serial sections.

They showed bean shaped nucleus and plump star-shaped cytoplasm; the invading
macrophages showed larger size and irregular shape.

Hapatocytes and macrophages were identified by arrows in Figure 4 as suggested by
the reviewer.

Minor Revisions:

1. Page 3 “Background” first sentence of the last paragraph: “cell related proteins”
were changed to “cell cycle related proteins”. It was a typing mistake.

2. Page 7, first sentence “only partially restituted p21….” was changed to
“completely restituted p21…” as indicated.

3. Page 7, second paragraph “Bcl 2 expression is highly induced….” was changed
to “Bcl 2 expression is slightly induced …” as indicated.

4. Figure 2 was modified as indicated.
5. Figure 3 was modified as indicated.

6. From our immunohistochemical analysis there is, predominantly, a trend to see a peri central vein (PCV) expression pattern of HO-1 in the liver after treatment. This is a common non-specific pattern in many hepatic damages; therefore we do not considered of special relevance in DAB hepatic treatment injuries.

Referee 2: Masato Noguchi

Major Revisions

1) The results of immunohistochemistry were added in the abstract. Page 2 line 19: “The immunohistochemical studies revealed the presence of macrophages surrounding foci of necrosis and nodular lesions in HR indicative of an inflammatory response. Furthermore, regenerating cells displayed changes in type, size and intensity of HO-1 immunostaining”.

2) Regarding to the heme degradation activity, we have previously demonstrated that DAB treatment induced HO1 at transcriptional and translational level, and that the level of induction persisted until the end of the intoxication protocol (6 months and 1 year) and as long as the oxidative damage lasted (Vazquez et al. 2002, reference 19 & Caballero et al 2004, reference 14).

The following paragraph was added in the discussion session, page 8, last paragraph: “HO-1 is an inducible and ubiquitous 32kDa isoform highly expressed in spleen and liver and normally found in very low levels in mammalian tissues (23). The regulation of its potent enzymatic activity depends primarily on the control of HO-1 expression at transcriptional level (23-25)”.

The following references were added in References (page 12):


The number of the rest of the references were modified

3) The following paragraph was added in the discussion session (page 9, last paragraph) in order to generalize our results to other pathological conditions as the reviewer suggested.

“HO-1 has also been identified as a key enzyme for the cytoprotection of many cell types. Increased expression of HO-1 has been shown to be protective in ischemia/reperfusion injury, in organ transplantation, in protection against renal and pulmonary injury, and in amelioration of adverse hemodynamic effects resulting from liver disease and portal hypertension [33,34,35]. Even more, it was recently reported that hepatitis C (HCV) infection increased HO-1
mRNA expression and protein levels in human liver cells and was suggested to likely represent a protective response against possible oxidative or other insult from HCV proteins [36]. Thus, Ghaziani et al [36] suggested that HO-1 importance extends beyond its function in the catabolism of heme”.

The following references were added in the reference session (page 12):


Minor Revisions:

1. Bilirubin (page 2, line 4 and page 3, last paragraph) was changed to biliverdin. It was a typing mistake.

2. Fig.1 “SDL” was changed to “SLD”. It was a typing mistake.

3. Fig.3 “P” labels were added. It was a mistake

Discretionary Revisions:

1. We agree with the reviewer that it could be very interesting to study longer periods of intoxication. This will be done in the future. However, as we established in *Materials & Methods*, section *Animals and Treatments*: “Animals with chemical induced hepatocellular carcinoma received the carcinogen during 89 days, when preneoplastic lesions were evident as previously reported (Caballero et al, 2004)””. And 78 days was selected “according to preliminary results demonstrating that optical proliferative morphological features were evident, indicative of a liver adaptive response by histological confirmation”. We considered this time point to be the most adequate as the start up point of our investigation, as after longer periods (100 days) adenomas were visible (Caballero et al, 2004).

2. CDK4 variation was not significant after the statistical analysis was performed for the whole group; therefore we decided not to check on Cyclin D1.
NOTE: On account of Figure 4 file size, we have to send it as two files, one with figure 4 a, b & c and the other (as figure 5) with figure 4 d, e & f. We appreciate if the editorial could reorganize the figure again.

We hope we have complied with all the reviewers concerns and that you find the paper suitable for publication in Biomed Central Cancer.

Best Regards,

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