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Role of reactive oxygen species in the early stages of liver regeneration in streptozotocin-induced diabetic rats

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Abstract

Diabetes mellitus is a risk factor for prognosis after liver resection. In previous work, we found a pro-apoptotic state in the diabetic rat liver. In this work, this was also observed 1 hour post-partial hepatectomy (PH) and resulted in a deficient regenerative response 24 hours post-PH. Treatment with insulin and/or Desferoxamine (DES) (iron chelator) or Tempol (TEM) (free radicals scavenger) was effective in preventing the liver reactive oxygen species (ROS) production induced by diabetic state. High levels of ROS play a role in hepatic lipid peroxidation in diabetes before and after PH, and lead to increased pro-apoptotic events, which contribute to a reduced regenerative response. This becomes of relevance for the potential use of antioxidants/free radical scavengers plus insulin for improvement of post-surgical recovery of diabetic patients subjected to a PH.

Keywords: *diabetes, reactive oxygen species (ROS), liver regeneration, apoptosis, insulin*

Introduction

Directly or indirectly, the liver is a major target of insulin action. The onset of diabetes is accompanied by the development of various biochemical and functional abnormalities in the liver [1]. Ohkuwa *et al* showed that the diabetic state induces hydroxyl radical (•OH) generation and correlates with the level of lipid peroxidation (LPO) [2]. In previous work [3], we demonstrated that diabetic state promotes a significant increase in •OH, which correlated with increased levels of LPO in liver tissue. Furthermore, hyperglycemia significantly increased the expression of mitochondrial Bax, cytosolic cytochrome c levels and caspase-3 activity leading to increased apoptotic index. Treatment of diabetic rats with Desferoxamine (DES) [4] or Tempol (TEM) [5] (free radical scavengers) significantly attenuated the increase of reactive oxygen species (ROS) and LPO and decreased the pro-apoptotic status by reducing mitochondrial Bax levels, cytosolic cytochrome c levels and caspase-3 activity, but failed to return them to their normal values. Insulin showed similar results, but with a complete normalization of caspase-3 activity and the apoptotic index. Overall, it was shown that, at least in part, •OH acts as a reactive intermediate that leads to liver apoptosis in a streptozotocin (STZ)-induced hyperglycemia model [3].

Diabetes mellitus is also considered a risk factor for prognosis after liver resection in patients with hepatocellular carcinoma; postoperative morbidity is more common among diabetic patients than among non-diabetic patients. Post-surgery mortality is higher in diabetic patients, as diabetes increases the probability

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of liver decompensation [6]. The post-surgical recovery mass depends on the regenerative ability of remnant liver. Several studies [7–9] suggest that insulin deficiency leads to distorted intracellular signalling pathways in the type 1 diabetic liver and, as a result, the regenerative response is deficient [10,11]. The mechanisms responsible for altered liver regenerative process found in diabetic state still remain to be characterized.

To address the impact of ROS produced in the early stages of partial hepatectomy (PH) on the proliferation index (PI) 24 hours post-surgery, we performed the treatments with DES and TEM on (STZ)-induced diabetic (SID) rats.

Materials and methods

Animals and experimental groups

Animal care and treatments were conducted in conformity with Institutional guidelines in compliance with National and International laws and policies (Expedient 6109/012 E.C. Resolution 267/02). To constitute the SID group, 3-month-old Wistar rats were injected intraperitoneally with STZ (Sigma) at a dose of 60 mg/kg. Control rats received the vehicle citrate buffer alone. Fifteen days after STZ injection, a time when the drug's hepatotoxic effects disappear [12], we proceeded to determine serum glucose and insulin. The diabetic state was defined as a threshold blood glucose >13.2 mmol/L. Blood glucose levels and body weight were measured to assess the diabetic state; thus, finding values similar to those previously published by our group [3].

Half the rats treated with STZ were also injected two times daily with insulin (30 UI/kg/day) (SID + I) until euthanasia. Insulin doses were adjusted in order to maintain glucose levels in the range of 6 to 9 mmol/L. Additional groups of SID rats were treated with DES (SID + DES) or with TEM (SID + TEM) for 15 consecutive days, or co-treated with insulin (SID + DES + I; SID + TEM + I). DES and TEM were used for studies of inhibition of ROS production. DES is an iron chelator that prevents the formation of •OH from hydrogen peroxide via inhibition of the Fenton and Haber–Weiss reactions [4]. DES (100 mg/kg bw, i.p.) was administered to rats, once a day, in saline solution, starting 15 days after injection of STZ and for 15 days. TEM is a stable piperidine nitroxide that permeates biological membranes, and reduces the formation or the effects of •OH by scavenging superoxide anions or by reducing intracellular $Fe+^2$ concentrations or by directly scavenging •OH [5]. TEM (20 mg/kg bw, i.v.) was administered to rats, once a day, in saline solution, starting 15 days after injection of STZ and for 15 days. The animals had unrestricted access to water and standard rat food and were maintained on a 12-hour light-dark cycle.

After 4 weeks, the animals were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and we performed a typical 65–70% PH [13] or a corresponding Sham (Sh). Then, PH and Sh were euthanatized 1 hour (PH 1h and Sh 1h), 5 hours (PH 5h and Sh 5h) or 24 hours (PH 24 h and Sh 24h) post-surgery. There were at least six animals per experimental group (n = 6). Liver samples were frozen for Western blot analysis, and liver slices were embedded in paraffin for histological examination. Liver morphology was assessed by analyzing 4-μm-thick cross-sectional serial sections after staining with hematoxylin and eosin. Sh surgery had no significant effect on any of the parameters examined.

Determination of reduced glutathione (GSH), oxidized glutathione (GSSG) and lipid peroxidation (LPO)

GSH and GSSG were determined in total liver homogenates according to the protocol described by Tietze [14], and GSH/GSSG ratio was calculated. LPO was measured as described by Ohkawa *et al* [15].

ROS detection, especially hydroxyl free radical

The *in vivo* measurement of •OH, a highly reactive free radical, is very difficult [4]. Thus, salicylic acid (SA) has been used as a trapping agent for detecting •OH *in vivo* [16,17]. For this experiment, 30 minutes after SA injection (100 mg/kg body weight, i.p.), a set of animals belonging to each experimental group was anaesthetized and euthanatized.

SA and 2,3-dihydroxybenzoic (2,3-DHBA) were measured according to the methods of Tsai *et al* [18] and Yamamoto *et al* [19], respectively, with modifications as in previous work [3]. The ratio of 2,3-DHBA to SA was obtained.

Western blot analysis

Samples were prepared as previously described [20]. Equal amounts of protein were migrated in acrylamide gels and blotted onto PVDF membranes. Antibodies used: anti-Bax, anti-Bcl-x_L, anti-cytochrome c, anti-cyclin D1 (Santa Cruz Biotechnology, CA) and anti-β-actin (Sigma). After incubation, membranes were again incubated with secondary antibodies such as IgG-peroxidase conjugates (Pierce), and the resulting bands were detected by enhanced chemiluminescence (ECL; Pierce) detection. Autoradiographs were obtained by exposing PVDF membranes to Kodak XAR film. Densitometry values were estimated using the Gel-Pro software (Media Cybernetics). **[AQ5]**

Caspase-3 activity was determined according to the manufacturer's instructions using an EnzChek™ caspase-3 assay kit (Molecular Probes, USA). Fluorescence was measured at λex = 360 nm and λem = 465 nm.

PI was assessed by performing an immunohistochemical study in the tissue fixed in formalin and embedded

in paraffin and detecting proliferating cell nuclear antigen (PCNA). Thus, we obtained the PI, which is the average of hepatocytes in G1, G2, S and M/1000 hepatocytes counted in 10 fields (objective: 40x and Ocular: 10x) [21]. Determinations were made in all experimental groups euthanized 24 hours post-surgery.

Statistical analysis

Data are shown as mean ± S.E.M. Statistical significance was determined by *t*-tests or ANOVA with post-hoc comparisons by Tukey's test. Probability values <0.05 were considered statistically significant.

Results

Table I shows body weight, serum levels of glucose and fructosamine and serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determined enzymatically (WienerLab, Argentina), in control, SID and SID-treated with insulin, before and after 1 hour and 5 hours of surgery. Prior to STZ injection, body weight of diabetic and control rats was similar, while the differences found were not statistically significant. Thirty days after STZ administration, body weight of diabetic rats was significantly lower than that of the control group. At this time, blood glucose levels were significantly increased in SID rats, as compared to control animals. Insulin treatment increased body weight and decreased blood glucose levels, reaching the control group values. Besides, as can be seen in Table I, no changes were observed in these parameters after PH. Serum levels of fructosamine were assessed with the aim of monitoring blood glucose levels control during treatment with insulin. As expected, every group treated with insulin showed fructosamine levels similar to the control group. Compared to control animals, serum ALT and AST activities were increased in the diabetic state. These results are consistent with the STZ model described by others and by us [3,22–24]. On the other hand, Di Domenico *et al* have described a significant increase in serum activities of ALT and AST after PH [25]. In accordance with their results, a significant increase in serum activities of both enzymes in all experimental groups was observed after surgery (see Table I).

Table I. Body weights and metabolic parameters at 1 and 5 hours after surgery in control, SID and SID + I groups.

	Control	SID	SID + I
Body weigth (gr)	440 ± 9	298 ± 9[a]	421 ± 13[b]
Blood glucose (mmol/L)			
Sh	6.31 ± 0.30	21.45 ± 9.29[a]	4.28 ± 0.29[b]
PH 1h	4.78 ± 0.99	22.88 ± 0.83[c]	3.70 ± 1.20[d]
PH 5h	6.02 ± 1.93	22.55 ± 2.8[e]	3.13 ± 0.69[f]
Fructosamine (μmol/L)			
Sh	192.5 ± 15.6	404.1 ± 57.2[a]	155.7 ± 15.5[b]
PH 1h	137.7 ± 4.6	276.9 ± 20.1[c]	175.9 ± 6.7[c,d]
PH 5h	221.9 ± 8.5	644.1 ± 25.2[e]	116.3 ± 5.5[e,f]
ALT (U/L)			
Sh	13.2 ± 6.9	74.4 ± 48.8[a]	32.45 ± 15.9[b]
PH 1h	52.3 ± 14.4[a]	130.3 ± 56.2[b,c]	124.8 ± 22.7[c]
PH 5h	54.1 ± 18.6[a]	69.1 ± 24.7	55.5 ± 21.8
AST (U/L)			
Sh	80.3 ± 13.9	312.2 ± 32.8[a]	186.47 ± 32.2[a, b]
PH 1h	86.6 ± 9.4	382.1 ± 85.6[c]	228.2 ± 89.9[d]
PH 5h	395.7 ± 89.9[a]	965.9 ± 11.8[b,e]	615.5 ± 53.4[e,f]

Control: vehicle; SID: streptozotocin-induced diabetes; SID + I: streptozotocin-induced diabetes treated with insulin; Sh: Sham; PH1h: partial hepatectomy 1 hour; PH5h: partial hepatectomy 5 hours; ALT: alanine aminotransferase; AST: aspartate aminotransferase. No changes were observed in body weight after partial hepatectomy. Values are mean ± S.E.M. (n = 4 animals per group). [a]$p<0.05$ vs Sh control; [b]$p<0.05$ vs Sh SID; [c]$p<0.05$ vs control PH1h; [d]$p<0.05$ vs SID PH1h; [e]$p<0.05$ vs control PH5hs; [f]$p<0.05$ vs SID PH5hs.

In order to determine the earlier time at which there were differences in the redox status of the cell, at two times post-surgery, 1 and 5 hours, we evaluated glutathione, which is the main redox buffer of the cell, by determining the ratio of GSH/GSSG as a key tool to assess the cellular redox state. We also determined LPO levels, which is commonly used as an indicator of oxidative stress in biological systems [14,15]. No significant differences were obtained among values from Sh animals studied 1 and 5 hours post-surgery (data not shown). Works from several laboratories, including our own [26–29], have shown that PH caused a diminution of the GSH/GSSG ratio, since there is a tendency to convert GSH to GSSG under oxidative stress at 1 and 5 hours after PH with respect to Sh rats (control group) (Figure 1A). The diabetic state significantly reduced the GSH/GSSG ratio as compared to Sh-control rats, while no further decrease was observed after PH. Treatment with insulin increased the GSH/GSSG ratio but without reaching the levels of Sh-control; a pattern similar to that of the control group was obtained when PH was performed (see Figure 1A).

In the same way, several lines of evidence, including our own, indicate that after PH [30–33], there is an increase in hepatic LPO levels. In the present study, we observed a similar increase of LPO levels at 1 and 5 hours after PH when compared to Sh rats in control group. The diabetic state (SID group) significantly increased the LPO levels in Sh, PH1h and PH5h, when compared to Sh-control rats. Insulin treatment prevented the increase of LPO levels in Sh-diabetic rats and a pattern similar to that of the control group was observed when PH was performed (see Figure 1A).

Based on the results obtained in the ratio of GSH to GSSG and LPO levels, the following studies were performed at 1 hour after PH, since this is the first time point at which we observed changes in cellular redox state; this would allow us to characterize the early events of the regeneration process, with an aim to determine its impact on the final process of proliferation.

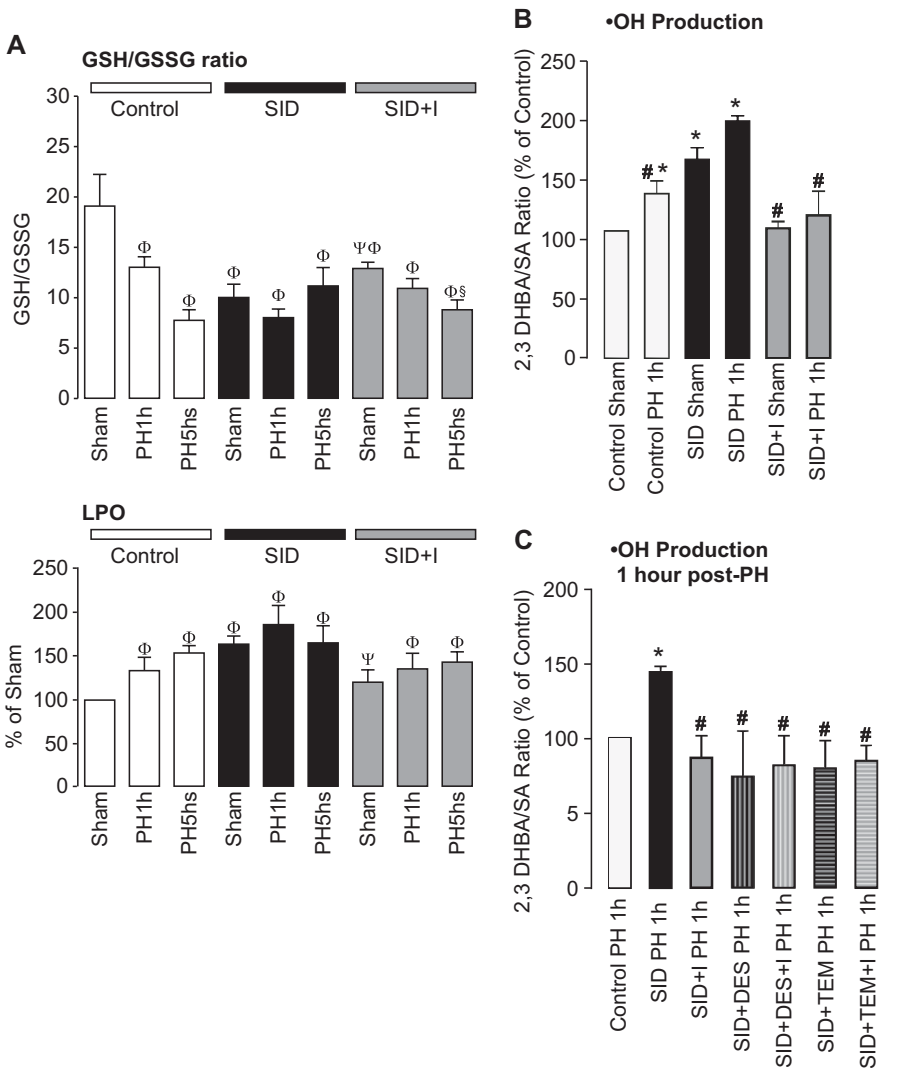

Figure 1. A) Determination of cellular redox state at 1 and 5 hours after surgery in control, SID and SID 1 I groups. GSH/GSSG ratio and lipid peroxidation (LPO) were determined in total liver homogenate of rats of all experimental groups. LPO was expressed as percentage of the Sham control. No significant differences were observed among the values obtained from Sham animals studied 1 and 5 hours post-surgery. Data are expressed as mean ± S.E.M., $n = 5$ for each experimental group. Φp < 0.05 vs Sham control; ψp < 0.05 vs Sham SID; §p < 0.05 vs Sham SID + I. B) Production of ROS, especially hydroxyl free radical (•OH) was determined as 2,3-DHBA/SA ratio. Data are expressed as percentage of Sham control. The experimental groups are: control, SID and SID + I, studied at 1 hour after Sham surgery or partial hepatectomy (PH). $^{*}p < 0.05$ vs Sham control; #$p < 0.05$ vs Sham SID. C) Production of ROS, especially hydroxyl free radical (•OH) determined as 2,3-DHBA/SA ratio analyzed 1 hour after partial hepatectomy (PH). Data are expressed as percentage of control PH1h. Data expressed as mean ± S.E.M., $n = 5$ for each experimental group. $^{*}p < 0.05$ vs control PH1h; #$p < 0.05$ vs SID PH1h.

In this study, ROS production (especially hydroxyl free radical) in the remnant liver after PH was evaluated. The PH increased liver ROS production (+30%) compared with the Sh-control group (Figure 1B). In a previous work, we have demonstrated the contribution of •OH in the production of LPO in the liver of SID rats [3]; in the present study, diabetic state (SID group) significantly increased the ROS production (especially hydroxyl free radical) in Sh when compared to Sh-control rats (see Figure 1B). After PH, the increase in the ROS production observed in SID rats in comparison with Sh-diabetic rats was not statistically significant. Insulin treatment prevented the increase in ROS production in Sh-diabetic rats and a pattern similar to that of the control group was observed when PH was performed (Figure 1B). Treatment with DES or TEM was effective in preventing the liver ROS production induced by diabetic state, reaching the values of the PH of control and the SID + I groups (Figure 1C).

We demonstrated in a previous work that in the early stages of liver regeneration after PH, changes occur in the expression of both pro-apoptotic Bax and anti-apoptotic Bcl-x_L proteins [34]. In addition, we showed that in the diabetic state there was a relative prevalence of Bax, which promotes cell death by apoptosis [3]. In this study, we showed that the PH significantly increased the mitochondrial Bcl-x_L expression in all experimental groups (control, SID and SID + I) (data not shown). The fate of the cells is largely dependent on the Bax/Bcl-x_L ratio [34]. We observed that in the diabetic

animals, after PH, the Bax/Bcl-x_L ratio was increased significantly versus control HP1h. Treatment with DES or TEM and/or insulin reduced the Bax/Bcl-x_L ratio (Figure 2A).

It is known that Bax protein promotes cell death via homodimerization, whereas heterodimerization with Bcl-x_L results in cell survival [34]. Disruption of the mitochondrial membrane by Bax homodimerization leads to the release of cytochrome c, which results in the activation of caspase-3 [35]. Therefore, we assessed the leakage of cytochrome c into the cytosol, as well as the activity of caspase-3. In all the hepatectomized groups studied, immunoblotting analyses showed that hyperglycemia increased the concentration of cytosolic cytochrome c (Figure 2B) in association with an up-regulation of caspase-3 activity (Figure 2C). In a previous work, we demonstrated that the diminution of LPO levels by antioxidant vitamin treatment during the early steps after PH produced a marked increase in the proliferation process [34]. In the present study, we analyzed PCNA level, which increases at 24 hours after PH in control group (Figure 3A). In SID rats, PCNA levels remained low, while the treatment with DES or TEM and/or insulin increased them; this fact

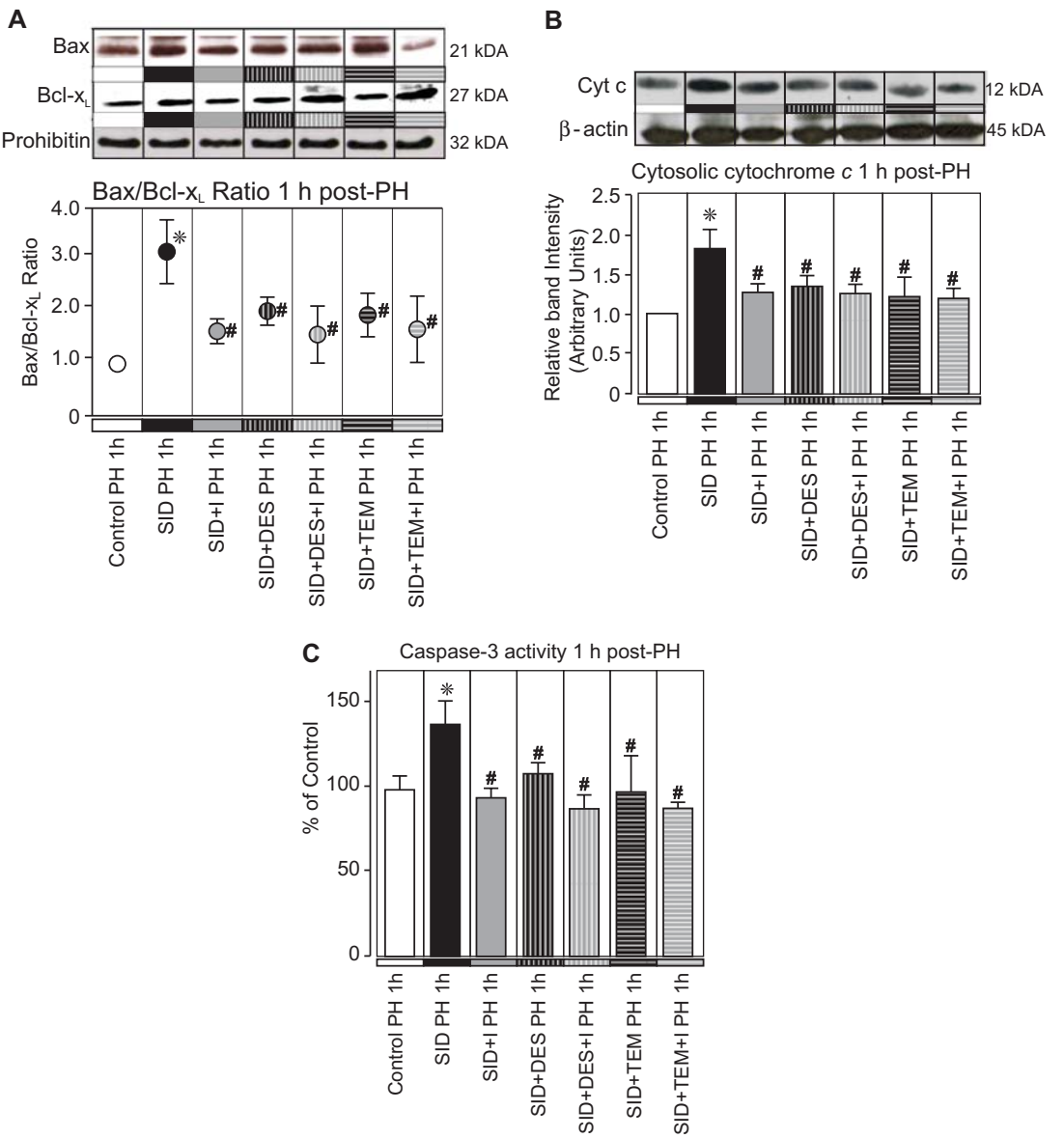

Figure 2. A) Mitochondrial Bax and Bcl-x_L protein expression. Typical example of Western blot is shown in the top panel for each experimental group. The accompanying graph represents mitochondrial Bax/Bcl-x_L ratio determined 1 hour post-PH in all experimental groups, expressed as percentage of the control PH1h group by the densitometry obtained for Bax and Bcl-x_L by Western blot analysis. Data are expressed as means ± S.E.M. for at least six rats for each experimental group: control, SID, SID + I, SID + DES, SID + DES + I, SID + TEM and SID + TEM + I, all of them under study 1 hour post-PH. *$p < 0.05$ vs control PH1h; #$p < 0.05$ vs SID PH1h. B) Cytosolic cytochrome c expression. Typical example of Western blot is shown in the top panel for each experimental group. The accompanying bars represent the densitometry expressed as Relative band Intensity (Arbitrary Units) from six separated animal sets. Data are presented as mean ± S.E.M. C) Determination of caspase-3 activity 1 hour post-PH. It was assayed fluorometrically. The bars represent activity expressed in percentage, considering control PH1h as 100%. Data are expressed as mean ± S.E.M. *$p < 0.05$ vs control PH1h; #$p < 0.05$ vs SID PH1h.

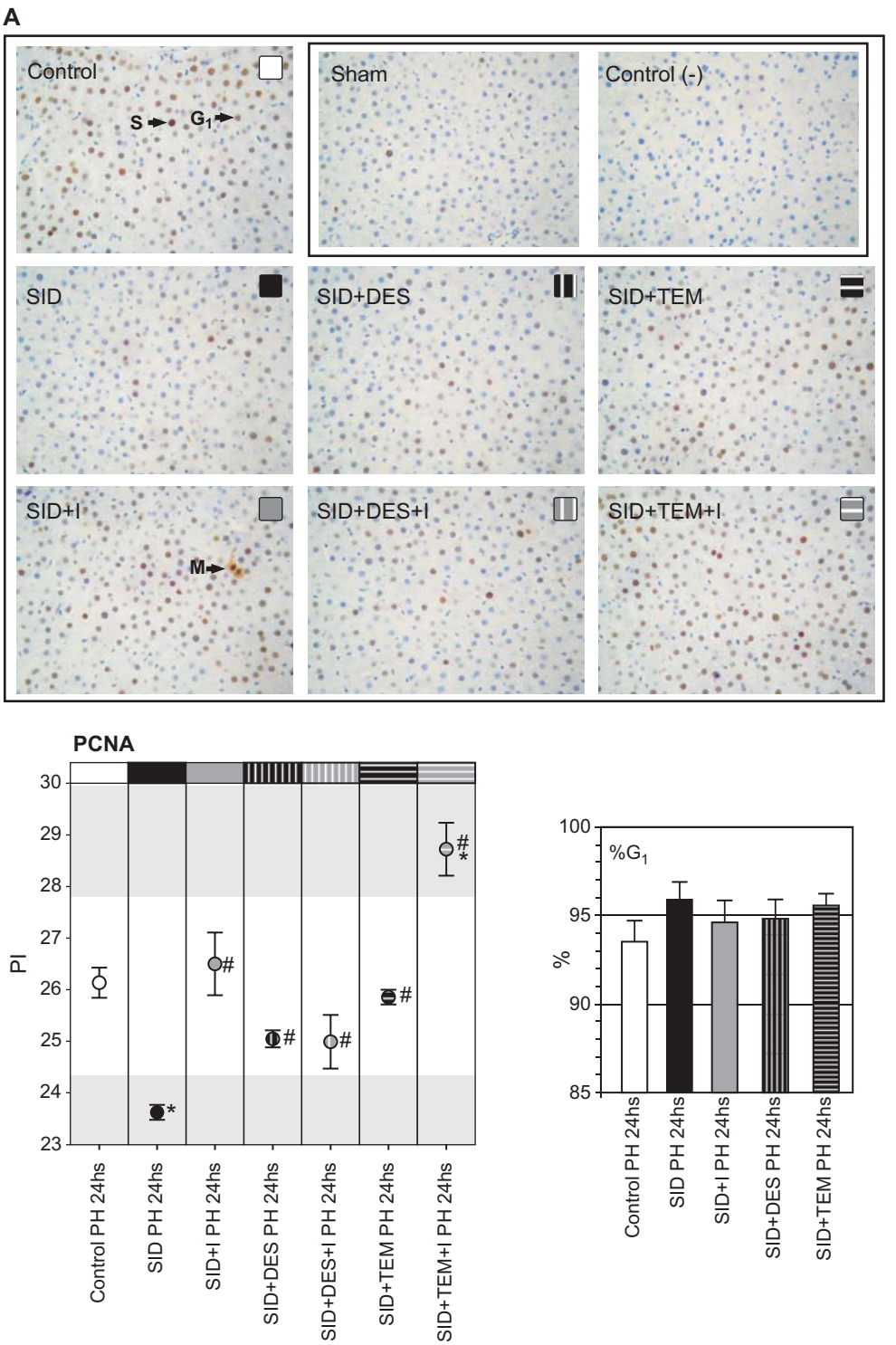

Figure 3. A) Light microscope images of PCNA detection 24 hours after surgery. Six animals were studied in each group. A negative control (no secondary antibody) is shown. Sham surgery (Sham), control, SID, SID + I, SID + DES, SID + DES + I, SID + TEM and SID + TEM + I, were studied 24 hours post-PH. Positive immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was evaluated by the brown-to-black reaction product that correlates with the different phases of the cell cycle. Typical colouration of several phases is presented: G1, S and M. (Objective: 40X and ocular 10X). Percentages of hepatocytes in G1 cell cycle phase assessed by immunohistochemical study are shown. We found a diminution in the proliferation index (PI) in SID group and an improvement of this parameter was achieved with insulin or antioxidants/free radical scavengers. No differences were observed between Sh-control, Sh-treated with insulin and/or antioxidants/free radical scavengers or their corresponding vehicles. PI, which is the average of hepatocytes in G1, G2, S and M/1.000 hepatocytes counted in 10 fields (objective: 40x and Ocular: 10x), was assessed by immunohistochemical detection of PCNA in tissue fixed in formalin and embedded in paraffin. Data are expressed as mean ± S.E.M. $^{*}p < 0.05$ vs control PH24hs; $\#p < 0.05$ vs SID PH24hs. B) The amount of cyclin D1 in nuclear extracts from livers of Sham and PH 24h after surgery of control, SID, SID + I, SID + DES and SID + TEM groups was measured by Western blot. Typical blot is shown in the top panel. The densitometric analysis of the bands is presented and expressed as Relative band Intensity (Arbitrary Units). Results are mean ± S.E.M. $^{*}p < 0.05$ vs control PH24hs; $\#p < 0.05$ vs SID PH24hs.

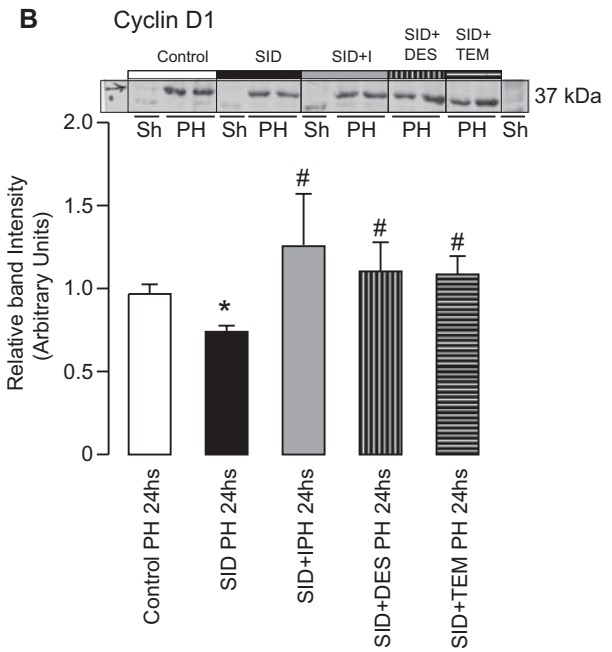

Figure 3. (*Continued*)

suggests that ROS produced by the hyperglycemia is not required for hepatocytes to enter into the S phase of the cell cycle. Moreover, the increases of PCNA obtained upon administration of TEM plus insulin were significantly higher than those obtained in the other experimental groups. Cyclin D1 expression, which plays a key role in cell cycle progression in hepatocytes post-PH [36], showed a reduced response in SID rats when compared to the control group (Figure 3B). The treatment with DES or TEM or insulin increased the expression of cyclin D1. It is evident that the presence of ROS produced by hyperglycemia results in a delay in the first cycle of hepatocyte proliferation.

Discussion

This study demonstrates the role of ROS in the cell proliferation/apoptosis balance in the early stages of the process of liver regeneration after a 70% PH and its impact on the PI 24 hours post-surgery in diabetic rats.

Previous studies of our group have shown that a selective increase in liver LPO in the early stages after PH could be a modulator of cell division by influencing the onset and cessation of mitosis in the regenerating liver [30,34,37]. Also, it has been reported that after PH, the increase in ROS production plays a significant role in the modulation of hepatocyte proliferation [32,38].

Diabetes is known to be a major disorder in which oxidative stress and free radical production have been implicated through several lines of evidence [39,40], and moreover, it is a pathology characterized by a deficient regenerative response [10,41].

In order to select the time to perform our studies, we analyzed GSH/GSSG ratio and LPO levels at 1 and 5 hours after PH in the liver of three experimental groups: control, SID and SID + I. Already at 1 hour post-hepatectomy, there was a redox imbalance due to surgery in the control and SID + I groups. The diabetic group showed an increased oxidative stress level before the PH. In this group, after PH, we did not observe any alterations either in the GSH/GSSG ratio or in the LPO levels. It is possible that hyperglycemia leads to a state of increased oxidative stress in the diabetic rats [1], which overlaps with the effect of surgery.

On the basis of these results, the following studies were performed at 1 hour after PH, a time when changes in the cellular redox state had already occurred. This allowed us to characterize the early events of the regeneration process, thus trying to determine its impact on the final process of proliferation.

Previously, we have demonstrated an increase in LPO in the liver remnant after PH studied, which is necessary for the proliferative process to occur normally [34,42]. In the present study, we found that PH increased liver •OH production in the control group, thereby demonstrating a contribution of •OH in the production of LPO in the liver of hepatectomized rats. It is known that in the diabetic state, there is a delay in the process of hepatic regeneration [8,10,41,43]. In a previous work, we have shown that •OH contributes in part to LPO observed in the diabetic state [3]. In the present study, we observed that oxidative stress estimated by LPO produced by the diabetic state masks the increase occurred after the PH (Figure 1). GSH levels could result in decreased activity of glutathione peroxidase, which catalyzes the decomposition of hydrogen peroxide into oxygen and water. This in turn increases the levels of hydrogen peroxide, which undergoes Fenton reaction and produces •OH radical. In this connection, Manna *et al* [44] have demonstrated, in SID, that intracellular glucose burden decreased the activities of antioxidant enzymes, including glutathione peroxidase and the GSH/GSSG ratio, and also increased LPO. Therefore, we aimed to modify the levels of ROS in diabetic animals before they were subjected to PH in order to study its impact on the proliferative process within 24 hours after PH. **[AQ6]**

To study the involvement of ROS in the up-regulation of Bax protein and consequent release of cytochrome c, which results in caspase-3 activation in the liver remnant after PH of SID rats, diabetic animals were treated with DES or TEM. The potent iron chelator DES [45] and the direct scavenger of free radicals, TEM, which has also been reported to reduce the formation of •OH by scavenging superoxide anions [5], showed a strong reduction of Bax/Bcl-x_L ratio, diminution of the release to cytosol of cytochrome c and inhibition of caspase-3 activity, thus establishing a clear connection between ROS production and the pro-apoptotic proteins studied. Our *in vivo* studies demonstrated that hyperglycemia leads to an increase in ROS production in rat liver, which tends to increase after PH. The treatment with

insulin reduced ROS production and the pro-apoptotic proteins evaluated here. Co-administration of both DES/insulin and TEM/insulin did not provide any additional beneficial effects compared to that obtained using DES or TEM or insulin alone.

It is well established that members of the Bcl-2 family are critical regulators of apoptosis in a variety of cell types and appear to be cell specific [46–49]. Bax/Bcl-x_L ratio determines cell survival or death after apoptotic stimuli. Bax protein has been shown to promote cell death via homodimerization, whereas heterodimerization with Bcl-x_L results in cell survival [34]. Our study demonstrates that there is an increased expression of Bax/Bcl-x_L ratio in the diabetic state after PH, while this ratio showed a diminution when compared to SID for all treatments (insulin, DES and/or TEM). We propose that during the diabetic state after PH, there is a relative prevalence of Bax, which promotes cell death by apoptosis. It has been demonstrated that induction of Bax protein and its translocation from the cytosol to the mitochondria lead to the release of cytochrome c, which results in caspase-3 activation, thus inducing apoptotic cell death [35]. Our data show that the up-regulation of Bax may play a key role in the increase of caspase-3 activity by releasing cytochrome c from mitochondria, therefore leading to an increase of the apoptotic state, which could be the cause of altered proliferation in diabetes.

Earlier studies had demonstrated that modifications of most of the stimuli involved in early stages post-PH might be modulators of cell division, influencing the initiation and cessation of mitosis in the regenerating liver [30,46,50].

In order to assess whether the pro-apoptotic events found in the SID group before surgery, which remained in the first hour after PH, have some impact on the outcome of the regeneration process, PI was assessed by PCNA detection, 24 hours after PH. It is known that the maximum proliferation level is observed at 24 hours after PH and decreases towards 48 hours [46,51]. As expected, SID group showed a diminution in PCNA in comparison with control group. The PCNA detection, an immunohistochemical technique, allows the differentiation of hepatocytes in various stages of the cell cycle. In the SID group, we observed a tendency of the hepatocytes to accumulate in phase G1 of the cell cycle (Figure 3A), a situation that was improved by treatments with insulin, DES and/or TEM. The decrease found in the PI of SID indicates a lower number of hepatocytes entering the cell cycle. Cyclin D1 plays a key role in cell cycle progression in hepatocytes after PH [36]. In the present study, a significant reduction of cyclin D1 was observed in SID group, as compared to control group, 24 hours after PH; this was improved by the treatments with insulin, DES and/or TEM. In SID rats, we found a decreased expression of cyclin D1 and a tendency of hepatocytes to accumulate in the phase G1 of the cell cycle. These results suggest that fewer hepatocytes are able to enter the cell cycle, thus accumulating in phase G1. Our model is characterized by insulin deficiency and, as we showed, by an increase of •OH, which would lead to DNA damage [3,44,52]. As it is known, the passage of the restriction point into phase S is ruled mainly by the ability to access growth factors and not to find damage in the DNA [36]. Our results suggest that the increase in ROS production could be implicated in the deficiency in the regenerative process observed in the SID group, since the groups treated with insulin and/or DES or TEM showed an improvement of proliferation process. Blood glucose levels found in diabetic groups treated with DES or TEM did not differ from those shown by the SID group at 24 hours post-surgery (data not shown), so that the improvement found in PCNA values of these groups is mainly attributed to the antioxidant capacity of these compounds, thus ruling out any hypoglycemic effect.

Interestingly, the groups SID + DES and SID + DES + I showed lower PIs than the groups SID + I, SID + TEM and SID + TEM + I. One possible hypothesis would involve the potent iron-chelating action of DES. It is known that iron is an essential element for a normal hepatic regeneration [53] and that during this process, complex mobilization of iron from different organs occurs [54]; therefore, any alteration in iron homeostasis may have implications in the regenerative profile. DES being a potent iron chelator, it could be affecting iron homeostasis, thereby opposing a major improvement of the process of proliferation. In this connection, it has been described that Fe chelators decrease ribonucleotide reductase activity, which is the rate-limiting enzyme involved in the conversion of ribonucleotides into deoxyribonucleotides (dNTPs) for DNA synthesis [55]. Moreover, several studies have shown that Fe chelation affects the expression of proteins critical for cell cycle progression [56,57]. In addition, Fe chelation can also induce the tumour suppressor protein p53 that transactivates the genes involved in cell cycle arrest and apoptosis [58,59]. In a previous work, we showed that the pre-treatment with antioxidant vitamins produces an increase in PI after PH in normoglycemic rats [22]. As TEM is an antioxidant that prevents either the generation or the effects of ROS consequently, its treatment improves the PI after PH in diabetic rats. Besides, it has been shown that insulin decreases hyperglycemia, leading to an attenuated ROS production, and consequently, decreases LPO [3,44]. These two facts support the finding that co-administration of insulin and TEM provides additional benefits on IP compared with that obtained using either insulin or TEM alone. SID group shows a decreased regenerative capacity while treatment with insulin, DES or TEM shows an increase in PI. This was confirmed by a decrease in cyclin D1 expression in SID and a tendency to the accumulation of hepatocytes in G1 phase. High levels of ROS play an important role in the increase of LPO

[AQ7] **[AQ8]**

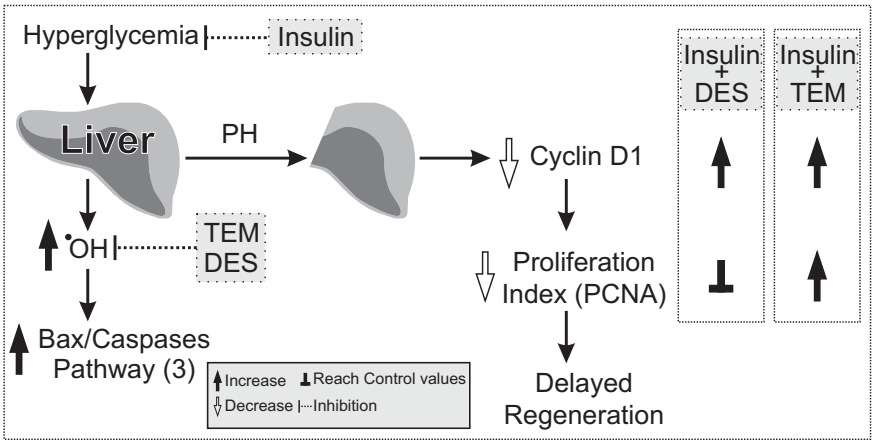

Figure 4. Diagram of the sequence of events in liver regeneration after partial hepatectomy (PH) in the diabetic state. STZ-induced hyperglycemia increases the ROS production, leading to an augmentation of Bax/Caspases pathway activity [3]. After 24 hours of PH, there is a diminution of the PI (determined by PCNA as is described in 'Materials and methods' section), which could lead to a delay in liver regeneration when compared to the events occurring in a normoglycemic state. Insulin decreases hyperglycemia, thus leading to an attenuated ROS production, and consequently, attenuates Bax/caspases pathway. Decrease in ROS production by Desferoxamine (DES) or Tempol (TEM) blocks Bax/caspases pathway. Overall, we consider that the treatment of STZ-induced diabetes with DES plus insulin increases cyclin D1 expression 24 hours after PH, leading to PI values that reach the normal ones. Importantly, the treatment of STZ-induced diabetes with TEM plus insulin increases cyclin D1 expression 24 hours after PH, leading to even higher PI values than those found in the control group.

in the liver observed in the diabetic state before and after PH, and ultimately lead to an increase in the number of pro-apoptotic events, thereby altering the delicate balance between the expression of pro- and anti-apoptotic proteins (Figure 4). Taken together, these results evidence a role of ROS induced by hyperglycemia in liver apoptosis, which may contribute to reduced regenerative capacity in diabetes. This knowledge becomes of relevance for the potential use of antioxidants/free radical scavengers plus insulin for improvement of post-surgical recovery of diabetic patients subjected to a PH.

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