Advances in Brief

Scavenging of Reactive Oxygen Species Leads to Diminished Peritoneal Tumor Recurrence

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Abstract

Previously, we demonstrated that RBCs inhibit the recurrence of perioperatively spilled tumor cells. The aim of this study was to identify on which RBC component(s) the inhibitory effect is based. By using a cell-seeding model in rats, the effect of RBC-related antioxidant scavengers [hemoglobin, catalase, and superoxide dismutase (SOD)] on peritoneal tumor recurrence was investigated. i.p. injection of hemoglobin caused 45% more tumor load ($P < 0.0001$). At least 40% inhibition of tumor recurrence was achieved with the use of catalase or SOD ($P < 0.05$). Combining SOD and catalase did not lead to additional inhibition of tumor recurrence. Inhibition of the overwhelming oxidative potential after surgical peritoneal trauma with the use of scavengers may lead to interesting new approaches for diminishing peritoneal tumor recurrence.

Introduction

Local or regional tumor recurrence of gastrointestinal carcinoma remains an important complication after potentially curative surgical resection (1). Prevention of this affliction remains the goal of many clinical and experimental studies (1–3). The results of these studies show a correlation between surgical peritoneal trauma and locoregional tumor recurrence (2, 3). Minimizing surgical trauma, through the use of laparoscopic surgery or other minimally invasive techniques, was shown to reduce peritoneal trauma and tumor recurrence (2, 3).

Excessive production of ROS[2] and related tissue injury play a fundamental role in a wide variety of disease processes (4, 5). Besides by chronic inflammatory diseases, ROS are also produced after surgical trauma (4, 6). The main producers of ROS are inflammatory cells entering damaged tissue after surgical trauma. The purpose of these cell products is to destroy invading organisms and damaged tissue. Despite this beneficial effect, the overwhelming oxidative potential can result in additional tissue destruction (4, 6).

After peritoneal surgery an acute inflammatory reaction occurs, even in the absence of any apparent bacterial contamination (7). We demonstrated recently that the inflammatory sequelae after abdominal surgery promote tumor recurrence and that this effect is mainly based on the cellular component of the inflammatory process (3, 8). In the same experimental model, we showed that RBCs, introduced in the peritoneal cavity after surgical trauma, effectively inhibited locoregional tumor recurrence (9). These studies were performed in a cell-seeding model and demonstrated that the inhibitory function of RBCs was primarily based on preventing adhesion of perioperatively

spilled tumor cells. Moreover, we demonstrated that RBC homogenates were equally effective, excluding the possibility of a mere steric hindrance of tumor cell adhesion.

The aim of this study was to identify which component(s) of the RBC homogenate could underlie the inhibitory effect of RBCs on tumor take. For this purpose, the effect of catalase (peroxide decomposition), hemoglobin (nitric oxide scavenger), and SOD (superoxide decomposition) were compared with the inhibitory effect of RBCs on peritoneal tumor recurrence.

Materials and Methods

Animals

Inbred rats of the WAG strain were obtained from Harlan-CPB (Zeist, the Netherlands). The rats were bred under specific pathogen-free conditions, kept under standard laboratory conditions (temperature, 20–24°C; relative humidity, 50–60%; 12 h light/12 h dark), fed with laboratory diet (Hope Farms, Woerden, the Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee for Animal Research of the Erasmus University Rotterdam.

Tumor Cell Line

CC531 is a moderately differentiated, weakly immunogenic colon adenocarcinoma induced in the WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium (10). CC531 tumor cells were cultured in RPMI 1640 supplemented with 5% FCS, 200 mM L-glutamine, and 10^5 units/l penicillin. All supplements were obtained from Life Technologicals BV (Breda, the Netherlands). Before use, cells were trypsinized (10 min at 37°C), centrifuged (5 min at 700 × g), resuspended in RPMI 1640, counted, and brought to a concentration of 0.5 million cells/ml. Viability was measured by trypan blue exclusion and always exceeded 90%.

RBC, Catalase, SOD, and Hemoglobin

To obtain RBC concentrate, the same procedure was followed as described previously (9). In short, WAG/Rij rats were exsanguinated by heart puncture. The blood was collected in a heparinized vial (heparin Leo; Leo Pharmaceutical Products, Weesp, the Netherlands). RBC concentrate was obtained by centrifugation of blood at 1000 × g for 10 min (Mistral 2000 i), removing the buffy coat and plasma. The pelleted RBCs were resuspended in RPMI 1640 to the original volume.

Catalase (3000 units/mg, from bovine liver) and hemoglobin (bovine, prepared from washed, lysed, and dialyzed erythrocytes) were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). SOD (5000 units/mg, from bovine erythrocytes) was purchased from Roche Diagnostics BV (Almere, the Netherlands). Before use, solutions containing the different scavengers in the desired concentrations were prepared in PBS and kept on ice.

In Vivo Experiments

Catalase and SOD Determination in RBCs Catalase. Pelleted RBCs were resuspended in 50 mM phosphate buffer (pH 7.0) to the original volume

and diluted 10 times in distilled water to lyse the RBCs at 37°C for 45 min. Next, the hemolysate was further diluted in phosphate buffer, added in a volume of 0.02–3 ml of 10.5 mM H_2O_2, and the time (s) was recorded to obtain a decrease in absorbance at 240 nm from 0.450 to 0.400. This value was used to calculate the amount of International Units of catalase/ml sample according to the formula: $17 \times 13.1/(time \times 0.02)$ (Ref. 11). We found (per ml of pelleted RBCs) 13563 ($\pm$ 1252) IU of catalase ($n = 8$); thus, 1.5 ml of RBC concentrate contained 9155 IU.

SOD. The RBC hemolysate was serially diluted in 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.01 mM ferricytochrome c, 0.05 mM xanthine, and 0.025 unit xanthine oxidase/ml. The SOD was assayed on the basis of its ability to inhibit the reduction of ferricytochrome c at 550 nm by superoxide anions generated by the xanthine-xanthine oxidase system (12). At pH 10.0, the SOD appeared about nine times more active and could be completely inhibited by 1 mM KCN. Pelleted RBCs contained 1096 units of SOD per ml, which meant that the RBC concentrate contained 750 units of SOD per 1.5 ml.

Operative Procedure and Tumor Scoring. Under ether anesthesia, the abdomen of the rats was shaved and cleaned with ethanol 70%. Laparotomy was performed using a midline incision, followed by i.p. inoculation of 0.5 million CC531 tumor cells. The abdomen was closed in one layer with silk 2-0 sutures (Braun, the Netherlands).

Three weeks postoperatively, the rats were sacrificed, and i.p. tumor load was scored as described previously (9) at the following sites: parietal peritoneum (at the site of the incision), omentum, liver, kidneys, retro peritoneum, and mesentery. The scoring ranged from 0 to 5 per site. A score of 0 meant there was no tumor growth, a score of 1 indicated an estimated tumor diameter of <0.5 cm, a score of 2 a tumor diameter between 0.5 and 1 cm, a score of 3 a tumor diameter between 1 and 2 cm, a score of 4 a tumor diameter between 2 and 3 cm, and a score of 5 an estimated diameter of >3 cm. For each rat, the scores were summarized and defined as total tumor load.

Effect of Catalase, SOD, and Hemoglobin on Peritoneal Tumor Recurrence. In this experiment, the inhibitory effect of RBCs on peritoneal tumor recurrence was compared with the effect of two major components carried by RBCs, *i.e.,* catalase and hemoglobin. After laparotomy, 0.5 million CC531 cells were injected into the peritoneal cavity together with 1.5 ml of PBS (control group, $n = 10$), with 1.5 ml RBC concentrate ($n = 10$), with 3000 units of catalase (in 1.5 ml of PBS, $n = 10$), or 0.5 mg of hemoglobin (in 1.5 ml of PBS, $n = 10$).

Next, the effect of a higher concentration of catalase was investigated. After laparotomy, CC531 cells were injected into the peritoneal cavity together with 1 ml of PBS (control group, $n = 10$), with 3000 units of catalase (in 1 ml PBS, $n = 9$), or with 6000 units of catalase (in 1 ml PBS, $n = 9$).

Finally, the effect of SOD alone and in combination with catalase was studied. The experimental groups received 0.5 million tumor cells together with 6000 units of catalase ($n = 8$), with 2000 units of SOD ($n = 8$), or with 6000 units of catalase and 2000 units of SOD ($n = 8$). To make up for total volume, 1 ml of PBS was added. The control group ($n = 5$) received 2 ml of PBS and 0.5 million of tumor cells.

Statistical Analysis

Statistical analysis was performed using the nonparametric Kruskal Wallis ANOVA to determine overall differences, followed by the nonparametric Mann Whitney U test to compare differences between groups.

Results

Effect of Two Major Components of RBCs *versus* Intact RBCs on Peritoneal Tumor Recurrence. After i.p. injection of tumor cells with RBCs, the inhibition of tumor recurrence was comparable (mean tumor load $\pm$ SE is 4.9 $\pm$ 2.0 in RBCs *versus* 19.3 $\pm$ 0.9 in control group, $P = 0.001$; Table 1; Fig. 1) with that as found previously (9). Administration of hemoglobin led to more tumor recurrence (28.3 $\pm$ 0.6, $P < 0.0001$ *versus* control), with statistically significant higher tumor diameters at four of six scored peritoneal sites. In the hemoglobin group, one rat suffered from overload peritoneal tumor load and was sacrificed before 3 weeks (this rat was excluded for statistical purposes). Significant differences in peritoneal tumor size were observed. In the hemoglobin group, 85% of the tumors were >3 cm, compared with 28% in the control and 3% in the RBC group (Fig. 1*B*).

When compared with the control, 3000 units of catalase inhibited tumor recurrence (10.5 $\pm$ 2.0 catalase, $P = 0.002$). Although this was a statistically significant inhibition, the effect of catalase on peritoneal tumor load was less than the inhibitory effect of RBCs ($P = 0.008$). In the catalase group, the majority of the tumors (40%) were between <0.5 and 2 cm in diameter, whereas in the RBC group, 50% was tumor free. Only 5% of the tumors in the catalase group were <3 cm in diameter.

To study whether increasing the amount of catalase would improve the inhibitory effect on tumor recurrence, 6000 units were administered i.p. Total tumor load in the control group of this experiment was similar to the control in the previous section (mean tumor load $\pm$ SE is 21.6 $\pm$ 1.5; Fig. 2*A*). However, in this experiment, the tumors were relatively large, *i.e.,* 42% of the tumors were <3 cm (Fig. 2*B*), whereas in the previous experiment, the control tumors were more heterogeneous in size (Fig. 1*B*). After i.p. injection of 3000 units of catalase, mean tumor load amounted to 12.3 $\pm$ 1.3 ($P = 0.004$ *versus* control). Tumor load did not decrease significantly using 6000 units of catalase (12.6 $\pm$ 2.4, $P = 0.003$ *versus* control).

Using more catalase also did not lead to a further decrease in tumor size. With 3000 units of catalase, 41% tumors were <0.5 cm, with 6000 units of catalase 43% (not significant).

Effect of Catalase and SOD on Peritoneal Tumor Recurrence. To investigate whether SOD could affect tumor recurrence as well, 2000 units of SOD were administered i.p. at the time of tumor seeding. SOD led to a significant reduction in tumor recurrence in comparison with the control, respectively, 12.6 $\pm$ 3.2 and 25 $\pm$ 2.9 ($P < 0.05$). The inhibitory effect of 6000 units of catalase was similar, as described in the previous section (Table 2). The combined administration of SOD (2000 units) and catalase (6000 units) showed a minor but not significant stronger inhibition of tumor recurrence (mean tumor score, 10.6 $\pm$ 2.3 in the catalase/SOD group; Fig. 3*A*; Table 2).

The sizes of tumor deposits in the antioxidant enzyme groups

Table 1 *Median tumor load (range) at different peritoneal sites*

Median tumor load and range at different abdominal sites in rats having undergone laparotomy followed by i.p. injection of tumor cells with PBS, RBCs, hemoglobin (0.5 g), or catalase (3000 units). p1 represents differences between rats receiving PBS (control) and RBCs, p2 represents differences between PBS and hemoglobin, p3 represents differences between PBS and catalase, and p4 represents differences between RBC and catalase.

Abdominal sites	PBS ($n = 10$)	RBCs ($n = 10$)	Hemoglobin ($n = 9$)	Catalase ($n = 10$)	p1	p2	p3	p4
Omentum	5 (4–5)	0 (0–5)	5 (5)	1.5 (0–4)	<0.0001	NS[a]	<0.0001	0.04
Liver	2.5 (2–4)	0 (0–2)	5 (5)	1.5 (0–3)	<0.0001	<0.0001	0.008	0.03
Kidney	3 (2–5)	1 (0–5)	5 (4–5)	3 (1–5)	0.004	0.002	NS	NS
Parietal peritoneum	5 (4–5)	1 (0–3)	5 (5)	1.5 (1–5)	<0.0001	NS	0.001	0.05
Mesentery	2.5 (1–4)	0 (0–4)	3 (2–5)	1.5 (0–4)	0.002	0.05	NS	NS
Retro peritoneum	1 (0–3)	0 (0–4)	5 (3–5)	1 (0–2)	0.05	<0.0001	NS	0.03
Total	19.5 (15–24)	2.5 (1–21)	28 (25–30)	12 (3–21)	0.001	<0.0001	0.002	0.008

[a] NS, not significant.

A

**Effect of RBC and Hb
on peritoneal tumor load**

B

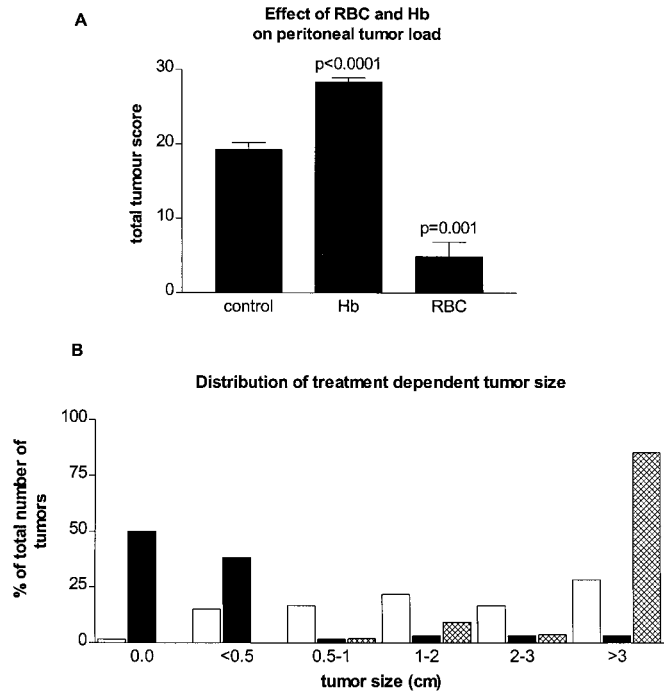**Distribution of treatment dependent tumor size**

Fig. 1. *A,* differences in peritoneal tumor load in rats receiving RBCs and hemoglobin compared with a control (PBS). The mean of the total tumor score is shown; *bars,* SE. *P*s, significant differences *versus* control group. *B,* distribution of treatment-dependent tumor size. □, control group; ■, RBS; ▨, hemoglobin.

varied greatly, whereas in the control group, mostly large tumors were scored (57% <3 cm). In the control group, 6.7% scored between 0 and <0.5 cm tumor size, in the SOD group 42%, and in the combined group 57% (Fig. 3*B*).

Discussion

The major finding of the present study is that the antioxidant enzymes SOD and catalase have been identified as the components that are (partially) responsible for the tumor-inhibiting function of RBCs. In a previous experimental study, we demonstrated that the inflammatory sequelae after surgery promote tumor recurrence and that this effect is mainly based on the cellular component of the inflammatory process (3, 8). Analysis of the cell population demonstrated the presence of >75% PMN. In ischemia reperfusion injury, where related inflammatory mechanisms are at play, most tissue trauma is inflicted by ROS (13–15). Scavenging of, or blocking, its production demonstrated that ROS significantly impede wound healing and graft survival after transplantation (14, 16, 17). Because PMN are a major source of ROS, similar PMN-related injury might play a role in surgical peritoneal trauma and tumor recurrence, and likewise, antioxidants may reduce tumor recurrence.

On the basis of previous results of a tumor-impeding effect of RBCs, we questioned which RBC component could underlie this effect. RBCs convey three major scavenging enzymes to diminish the toxic effects that can be caused by uncontrolled levels of superoxide radical anions (O_2^-) or hydrogen peroxide (H_2O_2). These are SOD, catalase, and glutathione peroxidase (18). Although hemoglobin is the principal oxygen carrier, it can scavenge nitric oxide radical as well (19).

We investigated the effect of hemoglobin and found that tumor recurrence was in fact increased compared with the control. Catalase inhibited tumor recurrence >40% in all experiments, showing the consistency of this effect *in vivo.* However, the peritoneal tumor load

remaining after catalase injection was significantly higher than after RBC injection. Although we detected 9000 units of catalase in RBC concentrate, we did not see a decrease in tumor recurrence when using a higher amount of catalase in the *in vivo* experiments. This indicates an additional tumor impeding effect brought about by RBCs that is not attained with catalase.

The affinity of catalase for its substrate hydrogen peroxide is low; in other words, catalase works efficiently only when the concentration of hydrogen peroxide is high. The finding that catalase is a potent inhibitor of tumor recurrence indicates that hydrogen peroxide is an important factor in enhancing peritoneal tumor recurrence. However, apparently the concentration of this ROS is low, and therefore glutathione peroxidase, another component of RBCs that reduces low concentrations of hydrogen peroxide in the presence of reduced glutathione with high efficiency (18), may be a better choice.

SOD, another antioxidant enzyme contained by RBCs, dismutates superoxide to peroxide and oxygen ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). Experiments investigating ischemia reperfusion injury use SOD to inactivate the tissue-traumatizing effect of superoxide (13–15). The results of these experiments show that neutralizing superoxide reduces tissue injury (15), enhances postoperative wound healing (14), increases bursting pressure of sutured small intestine (14), and increases graft survival after transplantation (13). In our experiment, 50% inhibition of tumor recurrence was observed by using this enzyme in excess (500 units present in RBC concentrate and 2000 units used in experiments). Apparently, the superoxide anion also promotes tumor recurrence. However, SOD alone and in combination with catalase was not more efficient than catalase alone.

The above-mentioned results illustrate that i.p. injection of hemoglobin stimulates tumor recurrence and that inhibition of hydrogen peroxide and superoxide-related tissue trauma diminishes
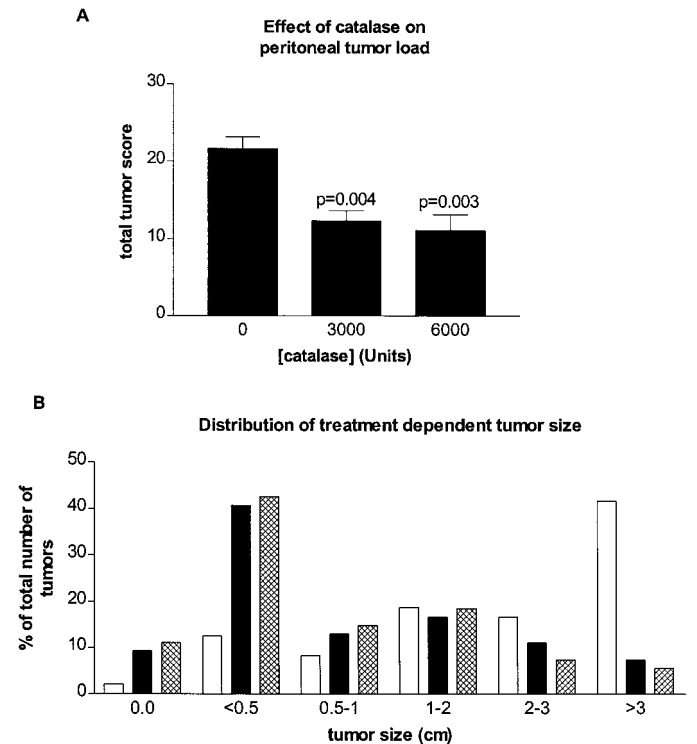
A

**Effect of catalase on
peritoneal tumor load**

B

Distribution of treatment dependent tumor size

Fig. 2. *A,* differences in peritoneal tumor load in rats receiving different concentrations of catalase. The mean of the total tumor score is shown; *bars,* SE. *P*s, significant differences *versus* control group (PBS). *B,* distribution of treatment-dependent tumor size. □, control group; ■, rats receiving 3000 units of catalase; ▨, rats receiving 6000 units of catalase.

Table 2 *Median tumor load (range) at different peritoneal sites*

Median tumor load and range at different abdominal sites in rats having undergone laparotomy followed by i.p. injection of tumor cells with PBS, catalase (6000 units), SOD (2000 units), or a combination of catalase and SOD (cat/SOD). p1 represents differences between rats receiving PBS (control) and catalase, p2 represents differences between PBS and SOD, and p3 represents differences between PBS and cat/SOD.

Abdominal sites	Control ($n = 5$)	Catalase ($n = 8$)	SOD ($n = 8$)	Cat/SOD ($n = 8$)	p1	p2	p3
Omentum	5 (3–5)	2.5 (0–5)	2 (0–5)	1 (1–3)	NS[a]	NS	0.003
Liver	4 (1–5)	1.5 (0–4)	1 (0–3)	1 (0–2)	NS	0.03	0.01
Kidney	4 (2–5)	0.5 (0–4)	2 (0–4)	1 (0–4)	0.007	0.02	0.01
Parietal peritoneum	5 (1–5)	0.5 (0–4)	2.5 (0–5)	4 (0–5)	NS	0.03	0.02
Mesentery	5 (1–5)	1.5 (0–4)	2 (0–5)	1 (0–5)	0.03	NS	0.03
Retro peritoneum	5 (2–5)	1 (0–5)	1.5 (0–5)	1 (0–5)	0.02	NS	0.04
Total	28 (14–30)	13 (1–25)	11.5 (1–26)	9 (2–24)	0.03	0.02	0.007

[a] NS, not significant.

tumor recurrence. Taken together, these results provide evidence for the role of another ROS, *i.e.,* the hydroxyl radical (OH·) as the real effector molecule in promoting tumor recurrence. Hydroxyl radicals are extremely reactive, unstable, and powerful free radicals compared with the relatively unreactive peroxide and superoxide (4, 5, 15). Catalyzed by iron, peroxide is converted to the hydroxyl radical in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^·$). On its turn, ferric iron is reduced to ferrous iron by superoxide in the Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^·$). This may explain why the combination of SOD and catalase did not lead to a better outcome than each antioxidant enzyme alone, and why hemoglobin, given as a source of iron, increased tumor recurrence. It is noteworthy that on a molar basis, we have administered the same amount of metal ($\sim$25 nmol) via hemoglobin (*i.e.,* Fe) and SOD (*i.e.,* Cu) that could participate in the Fenton reaction. Although direct generation of free hydroxyl radicals via SOD and hydrogen peroxide is not likely (20), the introduction of SOD always bears the risk of release of copper if the enzyme would degrade. The concomitant addition of compounds that can protect the enzyme from fragmentation may be

considered for this reason (21) and thus may even improve the inhibitory action of SOD on tumor recurrence. The production of the hydroxyl radical will ultimately lead to enhanced peritoneal tissue damage and, hence, enhanced tumor recurrence. Eliminating superoxide or hydrogen peroxide with SOD and catalase, respectively, prevents the production of the hydroxyl radical. Although glutathione peroxidase (together with reduced glutathione), based on its high efficiency to neutralize low hydrogen peroxide concentrations, may improve the inhibitory effect on tumor recurrence, a promising alternative approach may be the use of iron chelators. At the same time, using such compounds may circumvent the induction of an immunological reaction, which ultimately will occur against the exogenous antioxidant enzymes because of a difference in species between the donor and the recipient. However, it seems unlikely that such a reaction can explain the results of the present study because: (*a*) the development of a full-blown specific immune response needs more time; and (*b*) despite the source of the proteins under study was the same, *i.e.,* bovine, their effects on the inhibition of tumor recurrence were not.

In conclusion, we have shown that inhibition of ROS-mediated peritoneal damage with the use of scavengers leads to diminished tumor recurrence. A great deal of insight exists about ROS-mediated ischemia reperfusion damage and the prevention of this mechanism by scavengers. Recent studies proclaim improved results against tissue damage using chemically and biologically stable mimics of SOD and catalase (22). Inhibition of the overwhelming oxidative potential after surgical peritoneal trauma (or any other form of surgical trauma) with the use of scavengers may also lead to interesting new approaches for diminishing tumor recurrence.

Fig. 3. *A,* differences in peritoneal tumor load in rats receiving SOD (2000 units) and a combination of 6000 units of catalase and SOD (*cat/SOD*) compared with a control (PBS). The mean of the total tumor score is shown; *bars,* SE. *P*s represent significant differences *versus* control group. *B,* distribution of treatment-dependent tumor size. □, control group; ■, SOD; ▨, catalase and SOD.

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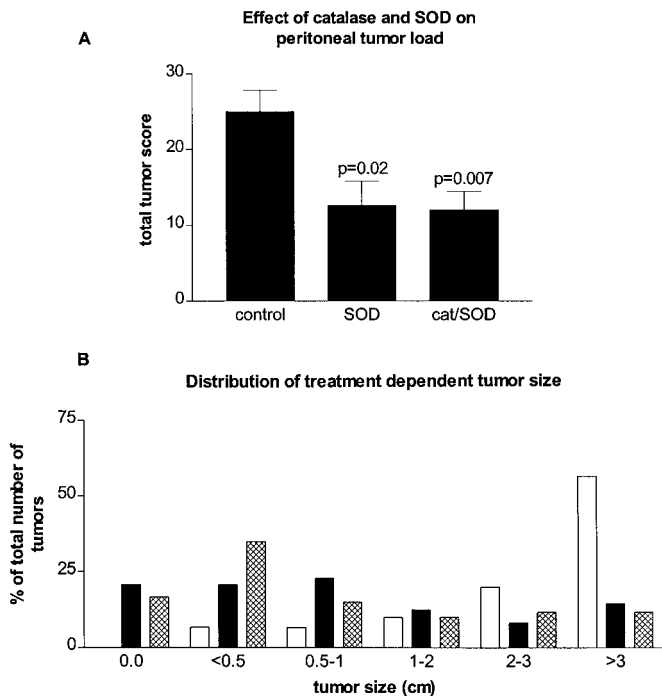
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