論文題目：Role of oxygen derived free radicals in the pathogenesis of coxsackievirus B3 myocarditis in mice

（マウスの実験的コクサッキーB3ウイルス性心筋炎の進展における活性酸素の関与）

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ROLE OF OXYGEN-DERIVED FREE RADICALS IN THE PATHOGENESIS OF COXSACKIEVIRUS B3 MYOCARDITIS IN MICE

Short title : Free radicals in myocarditis

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ABSTRACT

Objective : The aim was to test the role of oxygen-derived free radicals in the development of myocarditis. We investigated the effects of polyethylene glycol-conjugated superoxide dismutase (PEG-SOD, an enzyme catalyzing the conversion of O_2^- to H_2O_2) and polyethylene glycol-conjugated catalase (PEG-catalase, accelerating the reaction of H_2O_2 to H_2O and O_2), upon coxsackievirus B3 (CB3) myocarditis.

Methods : Two-week-old male C_3H/He mice were inoculated intraperitoneally with 10^3 plaque-forming units of CB3. PEG-SOD, $1x10^3$U/kg/day and PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day were administered subcutaneously daily on days 0 to 14. Treated groups were compared to the infected control.

Results : On day 7, there were no significant differences in pathologic scores among all groups. On day 14, however, the scores of cellular infiltration, myocardial necrosis and calcification were significantly lower in PEG-SOD, $1x10^3$U/kg/day treated group and PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group compared to the control. Futhermore, there were no signifficant differences in pathologic scores between only PEG-SOD treated group and a combination treatment with PEG-SOD plus PEG-catalase. There were no significant differences in the myocardial virus titers on day 7 among the three groups. On day 14, virus was not detected from the myocardium in the three groups.

Conclusions : The results suggest that superoxide anion is mostly responsible for myocyte injury in CB3 myocarditis in mice,

and that hydrogen peroxide formed as a consequence of dismutation of superoxide anion may not play a significant role in the development of myocarditis. Superoxide anion is one of the most important members in free radical-mediated injury in CB3 myocarditis in mice and the administration of PEG-SOD alone has therapeutic potential for clinical CB3 myocarditis equal to the combination of the two agents.

INTRODUCTION

Infiltration of the myocardium with inflammatory cells occurs during infection with a variety of viruses.[1,2] Usually, the infiltrate comprises mononuclear cells that are focal or diffusely scattered throughout the myocardium. Myofiber necrosis is an important feature of this lesion. Coxsackievirus B3 (CB3) is an enterovirus which can cause acute myocarditis in man.[1,2] In addition, viral myocarditis is considered a cause of dilated cardiomyopathy.[1-3]

There is no general agreement concerning effective therapy for viral myocarditis. Trials with steroids,[4,5] nonsteroidal antiinflammatory drugs,[6] immunosuppressive therapy,[7-9] β-blockers,[10] angiotensin converting enzyme inhibitors,[11] and other therapeutic modalities[12] have been attempted.

Superoxide dismutase (SOD), a potent scavenger of oxygen free radicals and catalase, accelerating the reaction of hydrogen peroxide to water and oxygen have been used for the treatment of several diseases, such as ischemic myocardial injury,[13-18] acute pancreatitis,[19-22] Behcet's disease,[23] influenza virus infection.[24,25] A recent study by Rezkalla et al.[11] suggested that the cardioprotective effects of angiotensin converting enzyme inhibitors against CB3 myocarditis may be due in part to their apparent ability to act as nonspecific antioxidants or specific scavengers of cytotoxic oxygen-derived free radicals, and that, therefore, oxygen free radicals may be involved in the pathogenesis of CB3 myocarditis. Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and polyethylene glycol-conjugated

catalase (PEG-catalase)[26,27] are known to have a plasma half-life in excess of 30 hours.

In a previous study,[28] we have shown that the administration of PEG-SOD, $1x10^3$U/kg/day reduced the severity of CB3 myocarditis. The present study was designed to determine

1) whether hydrogen peroxide formed as a consequence of dismutation of superoxide anion may play a role in myocyte injury and whether removal of hydrogen peroxide (H_2O_2) with catalase is necessary to attenuate cell damage in CB3 myocarditis in mice,
2) whether SOD alone may provide a level of protection that is equal to the combination of SOD and catalase.

To answer these questions, we examined the effects of PEG-SOD and PEG-catalase upon murine CB3 myocarditis.

METHODS

Infection protocol

The virus stock of coxsackievirus B3 (CB3) (Nancy strain : American Type Culture Collection) was prepared in culture of kidney cells of African green monkey in Eagle's minimum essential medium. Virus suspensions were centrifuged after the cytopathic effect had developed, and viral stock had a titer of more than 10^9 plaque-forming units/ml, determined by tissue culture.

Two-week-old, male, inbred, certified virus-free C3H/He mice (Shizuoka Laboratory Animal Center, Shizuoka) were used. The

animals were inoculated intraperitoneally with 0.1 ml of virus suspension containing 10^3 plaque-forming units of CB3. They were supplied with mother mice throughout the entire period.

All animals were cared for in accordance with the institutional policies and guidelines of Toyama Medical and Pharmaceutical University.

Treatment protocol (Figure 1)

PEG-SOD (Sigma Chemical Co., St. Louis) and PEG-catalase (Sigma Chemical Co., St. Louis) were administered subcutaneously daily at rotated sites, the actual dose for each experiment being calculated from mouse weight at the beginnig and middle of the experiment. The dosage of PEG-SOD was $1x10^3$U/kg/day, and that of PEG-catalase $1x10^3$U/kg/day. These doses were chosen based on previous reports.[14,16,17,20,26,27]

Ninety mice were randomly selected to no treatment (n=30), to treatment with PEG-SOD, $1x10^3$U/kg/day (n=30), and to treatment with PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day (n=30). Mice in the untreated group were injected subcutaneously with 0.1 ml of saline during the treatment period. Starting simultaneously after the virus inoculation, treatment was administered for 14 days. The mice were observed daily, and necropsy was performed immediately on those mice found dead.

Ten mice in each group were weighed and killed on day 7 by bleeding from the retroorbital plexus. Heart, liver and lungs were isolated and weighed aseptically. They were processed for virologic and pathologic studies. Mice surviving to the end of treatment period were also weighed, sacrificed and necropsied.

Heart, liver and lung were isolated and weighed. The organs were processed for virologic and pathologic studies.

To avoid the postmortem changes and to match the time course, pathologic study was performed only upon the mice sacrificed on days 7 and 14.

Additional control groups of uninfected mice, treated for 14 days with saline (n=5), $1x10^3$U/kg/day of PEG-SOD (n=5), $1x10^3$U/kg/day of PEG-SOD plus $1x10^3$U/kg/day of PEG-catalase (n=5) were also examined.

Pathologic study

Tissues (heart, pancreas, thymus, spleen, lungs, liver, kidney and psoas muscle) were processed by standard methods, embedded in paraffin, cut into 5μm thick sections, and stained with hematoxylin-eosin. Myocardial sections were graded by two of the authors (Y.H. and C.K.), blinded to the respective treatment groups, for the severity of cellular infiltraion, necrosis and calcification of the ventricles. The mean value was cited.

The pathologic criteria for grading of the severity of infiltration, necrosis or calcification were :

Grade 1 (mild)	:	one or two small foci
Grade 2 (slight)	:	several small foci
Grade 3 (moderate)	:	multiple small foci or several large foci
Grade 4 (severe)	:	multiple large foci
Grade 5 (very severe)	:	diffuse infiltration

necrosis or calcification

The other organs were evaluated for evidence of viral or other pathologic lesions.

Virological study

For infectivity assays, hearts were removed aseptically, weighed, and homogenized in 2 ml of phosphate buffered saline. After centrifugation at 1,500 rpm for 15 min, virus titers in the supernatants were determined by the plaque assay method.

In brief, African green monkey kidney cells were suspended at a concentration of $1x10^6$/ml in Eagle's minimum essential medium with 5% fetal calf serum plus 100 mg/ml of penicillin and streptomycin in six well plates and allowed to grow for 2 or 3 days at 37^oC in 5% CO_2. After adsorption, the cells were overlaid with 3 ml of Eagle's minimum essential medium containing 5% fetal serum and 1% methyl cellulose. After 2 days of incubation at 37^oC in a humidified atmosphere containing 5% CO_2, the cells were fixed with acetic acid and methanol (at a ratio of 1:3) and stained with 1% crystal violet; plaques were then counted with an inverted microscope.

Statistics

Heart-to-body, lung-to-body and liver-to-body weight ratios were analyzed by one way analysis of variance with multiple sample comparisons, comparing treated group versus control group on days 7 and 14. One way analysis of variance with multiple sample comparisons was used to evaluate difference in the pathologic scroes. Kaplan-Meier's method was used to determine

the significance of differences in survival rates. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Infection with CB3 produced a similar and pathologic picture to that reported previously.[8,9,12,29] In brief, three days after the virus inoculation, the mice appeared ill; some developed coat ruffling, weakness and irritability. Grossly, the myocardium had pale yellow patches that correlated with the inflammation, necrosis and calcification seen microscopically.

Mortality (Figure 2)

Two mice in the control group, 10 in PEG-SOD, $1x10^3$U/kg/day treated group, 6 in PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group had died by day 14; the survival rate on day 14 was 90.0% (18/20) in control group, 50.0% (10/20) in PEG-SOD, $1x10^3$U/kg/day treated group, 70.0% (14/20) in PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group. The difference between control group and PEG-SOD, $1x10^3$U/kg/day treated group was significant.

There was no death of uninfected mice in three groups throughout the entire period. And PEG-SOD and PEG-SOD plus PEG-catalase did not affect body weight gains in uninfected mice.

Cardiac pathology (Figures 3, 4 and 5)

On day 7, there were no significant differences in the score

of cardiac pathology (infiltration, necrosis and calcification) among the three groups.

On day 14, the scores of cellular infiltration, myocardial necrosis and calcification were significantly lower in PEG-SOD, $1x10^3$U/kg/day treated group and in PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group compared to the control.

No abnormalities were found in the heart of uninfected mice in the three groups.

Pathology of other organs

Pancreatitis, probably virus-induced, was noted in mice in each infected group. No viral lesions were noted in the thymus, lung, liver, kidney, spleen or muscle. There were no significant changes in the incidence of congestion of lung and liver in three infected groups.

No abnormal findings were observed in the pancreas, thymus, lung, liver, kidney, spleen or muscle in three uninfected groups.

Heart, Lung and Liver weights (Table 1)

On day 7, there were no significant differences in heart, lung and liver weights to body weight ratios. Similar results were also obtained on day 14.

Myocardial virus titers (Table 2)

There were no significant differences in the myocardial virus titers on day 7 among the three groups. On day 14, the virus was not detected from the myocardium in the three groups.

DISCUSSION

The results of present study clearly demonstrated that the severity of myocardial lesions of CB3 myocarditis in PEG-SOD, $1x10^3$U/kg/day and PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated groups were significantly lower compared to the control. Myocardial virus titers did not differ significantly in all groups. Furthermore, there were no significant differences in heart, lung and liver weights to body weight ratios among all groups. This finding may be of great value for understanding the pathogenesis of CB3 myocarditis.

There has been a substantial number of experimental studies testing the efficacy of superoxide dismutase (SOD) and catalase on several diseases.[13,17,24,25] Especially, many investigators in the fields of inflammation have expressed a growing awareness to oxygen-derived free radicals as a cause of tissue injury; particular attention has been focused upon the pivotal deleterious effect of free radicals in the pathogenesis of influenza virus infection,[24,25] pancreatitis,[19-22] and other inflammatory diseases.[23] Westlin and Mullane[30] suggested that the cardioprotective effects of angiotensin converting enzyme inhibitors that contain a sulfhydryl group (such as captopril and zofenopril) may be due in part to their apparent ability to act as nonspecific antioxidants or specific scavengers of cytotoxic oxygen-derived free radicals. Also, Rezkalla et al.[11] suggested that the postulated oxygen free radical scavenging properties of captopril might be beneficial in acute CB3 murine

myocarditis.

Huber and Saifer[31] reported that unmodified SOD was removed from the serum with a half-time of about 6 min in mice. Unfortunately, this short plasma life limits the clinical usefulness of SOD as free radical scavengers. The attachment of polyethylene glycol (PEG) to SOD and catalase results in an extension of the plasma half life and in the suppression of immunogenicity of the conjugates. Viau[27] reported that PEG-SOD administered intramuscularly remained at peak concentrations for 24 hours and stayed in the circulation for 8 days. Similarly, they reported that the blood-circulating life of catalase was increased from a few minutes to 3 days through the attachment of PEG to the enzyme.

Neutrophils and macrophages are known to produce superoxide free radicals (O_2^-) and hydrogen peroxide (H_2O_2),[32,33] which normally are involved in the killing of ingested or invading microbes. However, the activated oxygen can also cause tissue injury under conditions such as ischemic reperfusion and inflammatory diseases. Because the inflamed myocardium of viral myocarditis is associated with an intense leukocyte infiltrate,[1,2,29] there may be a possibility that oxygen radicals generated from these mononuclear cells could contribute to additional myocyte damage.

In the previous study,[28] we have shown that the administration of PEG-SOD, $1x10^3$U/kg/day reduced the severity of myocardial lesion of CB3 myocarditis. The study, however, did not attempt to discern the relative roles of the various oxygen species in CB3 myocarditis in mice. Both O_2^- and H_2O_2 are

theoretically capable of producing myocellular damage in themselves. In addition, O_2^- and H_2O_2 can react to generate the hydroxyl radical ($\cdot OH$), an oxidant considerably more potent than either of its precursors. Consequently, accumulation of O_2^- or H_2O_2 could produce myocardial dysfunction either directory, via the cytotoxic action of these species, or indirectory, via $\cdot OH$ generation. Administration of PEG-SOD plus PEG-catalase may help to discern these two mechanisms because this combinaton would not interfere with injury caused directoly by O_2^- or H_2O_2 but it should attenuate the toxic action of $\cdot OH$. Such information would not only help to elucidate the mechanisms of radical-mediated damage but may also have therapeutic implications. Both SOD and catalase are highly specific enzymes; SOD acts only by catalyzing the dismutation of O_2^- to H_2O_2 and O_2, while catalase accelerates the conversion of H_2O_2 to H_2O and O_2.

It is generally accepted that a biphasic disease process results when mice are infected with CB3.[1,2,29] During the acute phase, viral replication in the myocardium results in myocardial necrosis with inflammation during the first week. After the virus has been eliminated from the myocardium, a chronic inflammation results in progressive myocyte damage. There is a suggestion that the chronic phase results from cell-mediated immune response to a neoantigen that developed during the acute phase of illness.[1,2,8,9,29] In addition to the documented immunopathologic mechanisms,[8,9,29] it was suggested, in this study, that cardiac lesions of CB3 myocarditis in PEG-SOD, $1x10^3$U/kg/day and PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase,

$1x10^3$U/kg/day treated groups were less severe than those of untreated group; treatment with PEG-SOD or PEG-SOD plus PEG-catalase did not affect clearance of the virus from the myocardium. Accordingly, oxygen free radicals generated from infiltrating leukocytes and macrophages in the inflamed myocardium may contribute to the further development of CB3 myocarditis.

Two possible mechanisms of which treatment of PEG-SOD aggravated mortality might have been considered. One is the toxicity of PEG-SOD itself. The other is aggravation of virus-induced at extracatiac sites, such as pancreatitis.

The limitations of the present study are as follows. Although we had already examined the serial changes in lymphocyte subsets in the heart of C_3H/He mice inoculated with CB3,[29] it might be necessary to demonstrate macrophages and neutrophils in the inflamed myocardium. However, even in conventional hematoxylin-eosin stainings, these mononuclear cells were well distinguished. Furthermore, in this study, we might have discussed the possibility of treatment over shorter period. Also it might be necessary to initiate treatment at the onset of myocardial inflammation rather than at the time of infection.

In conclusion, superoxide anion is one of the most important members in free radical-mediated injury in CB3 myocarditis in mice, and that the administration of PEG-SOD alone has therapeutic potential for clinical CB3 myocarditis equal to the combination of PEG-SOD plus PEG-catalase.

ACKNOWLEDGEMENTS

This work was supported by research grants of Japanese Heart Foundation, Education Fellowship of Toyama Med. & Pharm. Univ., Iwaki Fellowship, Japanese Education of Science and Welfare (Nos. 01570478 and 03670445) and Uehara Memorial Foundation.

KEY TERMS

Myocarditis

Coxsackievirus B3

Oxygen free radicals

Superoxide dismutase

Catalase

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FIGURE LEGENDS

Figure 1 Schematic diagram of experimental design

PEG-SOD (polyethylene glycol-conjugated superoxide dismutase) and PEG-catalase (polyethylene glycol-conjugated catalase) were administered subcutaneously daily on days 0 to 14. The dosage of PEG-SOD was $1x10^3$U/kg/day, and that of PEG-catalase was $1x10^3$U/kg/day.

Figure 2 Plots of survival

Two mice in the control group, 10 in PEG-SOD, $1x10^3$U/kg/day treated group, 6 in PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group had died by day 14. The difference between control group and PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group was significant.

Figure 3 Results of cardiac pathology on day 7

There were no significant differences in the score of cardiac pathology among the three groups.

Figure 4 Results of cardiac pathology on day 14

The scores of cellular infiltration, myocardial necrosis and calcification were significantly lower in PEG-SOD, $1x10^3$U/kg/day treated group and PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group compared to the control.

Figure 5 Sections of myocardium

Fourteen days after virus inoculation, marked inflammatory cell infiltration and extensive myocardial necrosis were seen in a mouse of the control group (A). On the other hand, infiltration and necrosis were less severe in the heart of a mouse in PEG-SOD, $1x10^3$U/kg/day treated group (B) and in the heart of a mouse in PEG-SOD, $1x10^3$U/kg/day plus PEG-catalase, $1x10^3$U/kg/day treated group (C).

Hematoxylin-eosin, x380.

Table 1. Heart, lung and liver weights at day 7 of infection

	CONTROL (n=10)	PEG-SOD $1x10^3$ (n=10)	PEG-SOD $1x10^3$ (U/kg/day) PEG-catalase $1x10^3$ (U/kg/day) (n=10)
BW (g)	10.4±1.1	10.7±0.8	10.8±0.4
HW/BW ($x10^{-3}$)	6.8±0.8	6.3±0.8	7.7±1.1
LuW/BW ($x10^{-3}$)	9.3±1.3	9.6±1.8	13.2±4.2
LiW/BW ($x10^{-2}$)	5.0±0.5	4.7±0.6	4.8±0.7

(mean ± S.D.)

BW = body weight, HW/BW = heart weight / body weight, LuW/BW = lung weight / body weight, LiW/BW = liver weight / body weight.

Table 2. Virus titers in the heart (plaque-forming units/mg tissue)

	CONTROL	PEG-SOD $1x10^3$	PEG-SOD $1x10^3$ (U/kg/day) PEG-catalase $1x10^3$ (U/kg/day)
Day 7	$1.7\pm1.2x10^4$ (n=5)	$6.7\pm5.2x10^3$ (n=5)	$1.6\pm0.7x10^4$ (n=5)
Day 14	0 ± 0 (n=5)	0 ± 0 (n=5)	0 ± 0 (n=5)

(mean ± S.D.)

Figure 1.

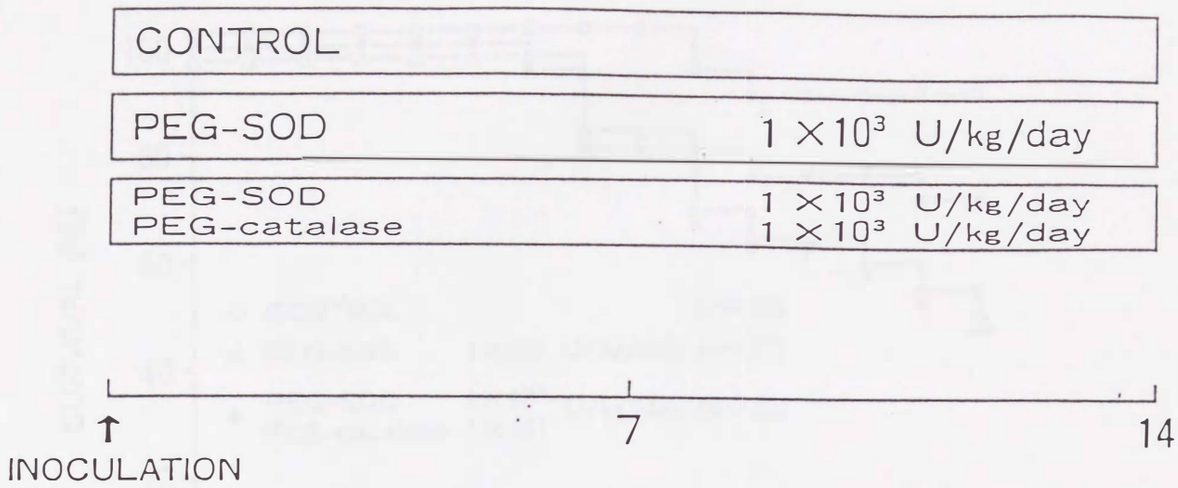

CONTROL
PEG-SOD
1 × 10³ U/kg/day
PEG-SOD
1 × 10³ U/kg/day
PEG-catalase
1 × 10³ U/kg/day
7
14
INOCULATION
DAYS

Figure 2.

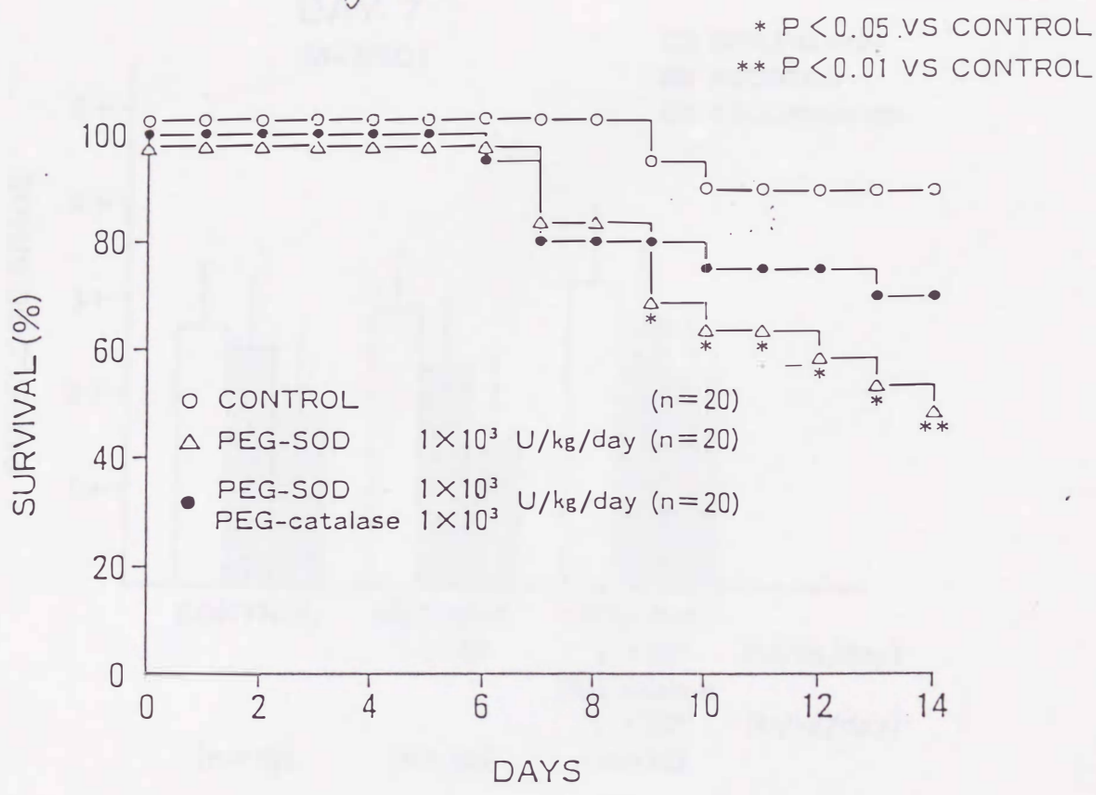

* P < 0.05 VS CONTROL
** P < 0.01 VS CONTROL
SURVIVAL (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
DAYS
○ CONTROL (n=20)
△ PEG-SOD 1×10³ U/kg/day (n=20)
● PEG-SOD 1×10³ U/kg/day
PEG-catalase 1×10³ (n=20)

Figure 3.

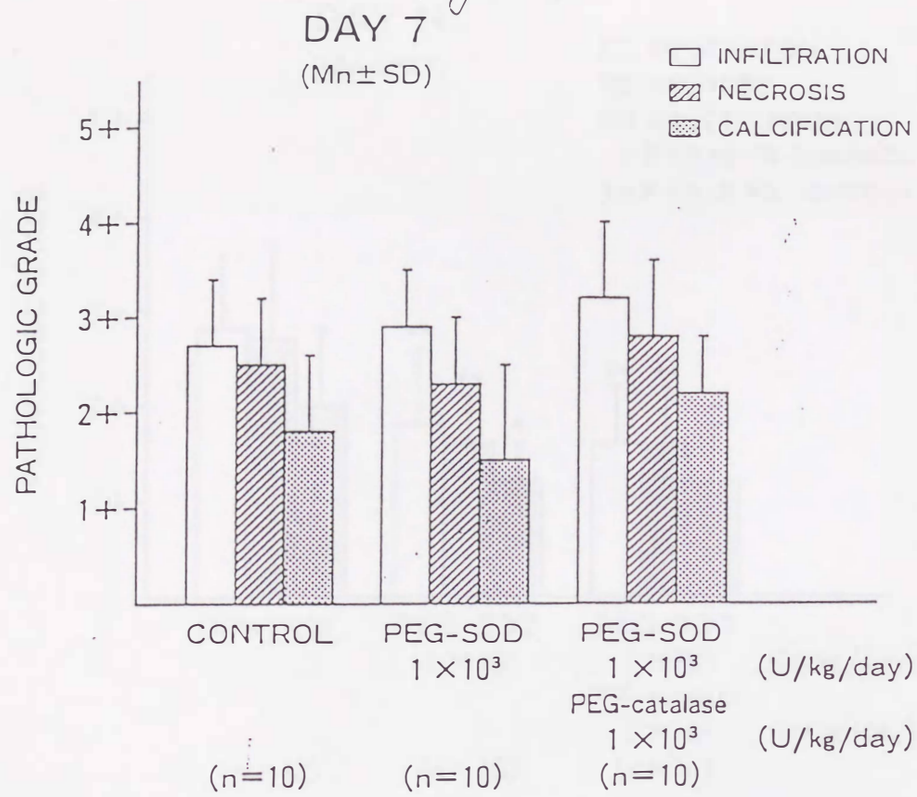

DAY 7
(Mn±SD)
INFILTRATION
NECROSIS
CALCIFICATION
PATHOLOGIC GRADE
5+
4+
3+
2+
1+
CONTROL
PEG-SOD
1×10³
PEG-SOD
1×10³
(U/kg/day)
PEG-catalase
1×10³
(U/kg/day)
(n=10)
(n=10)
(n=10)

Figure 4.

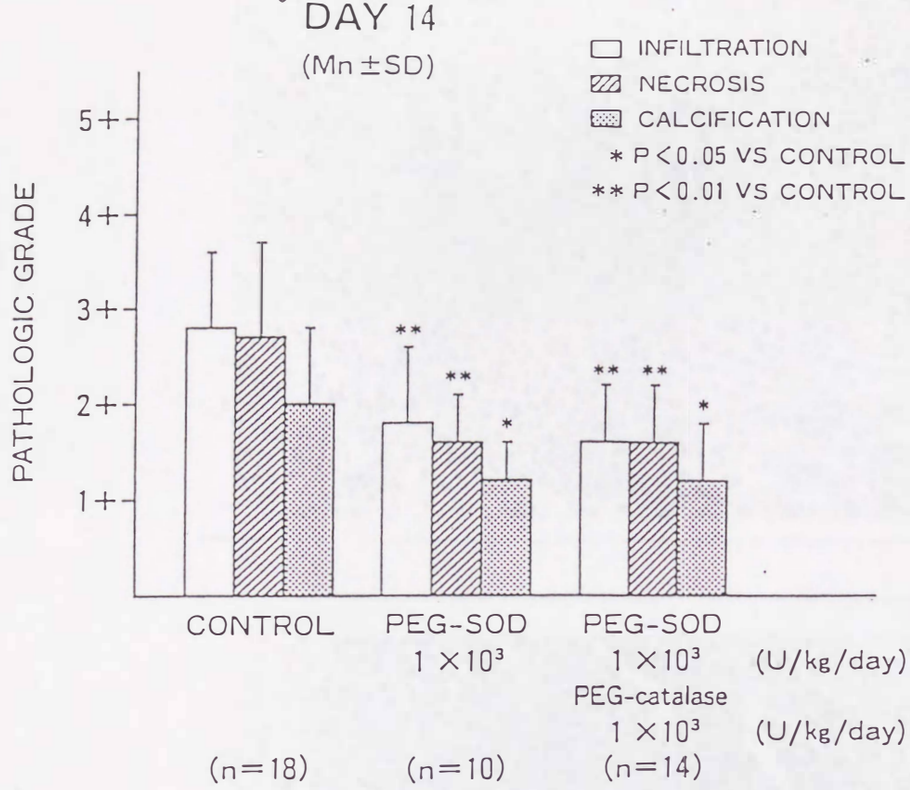
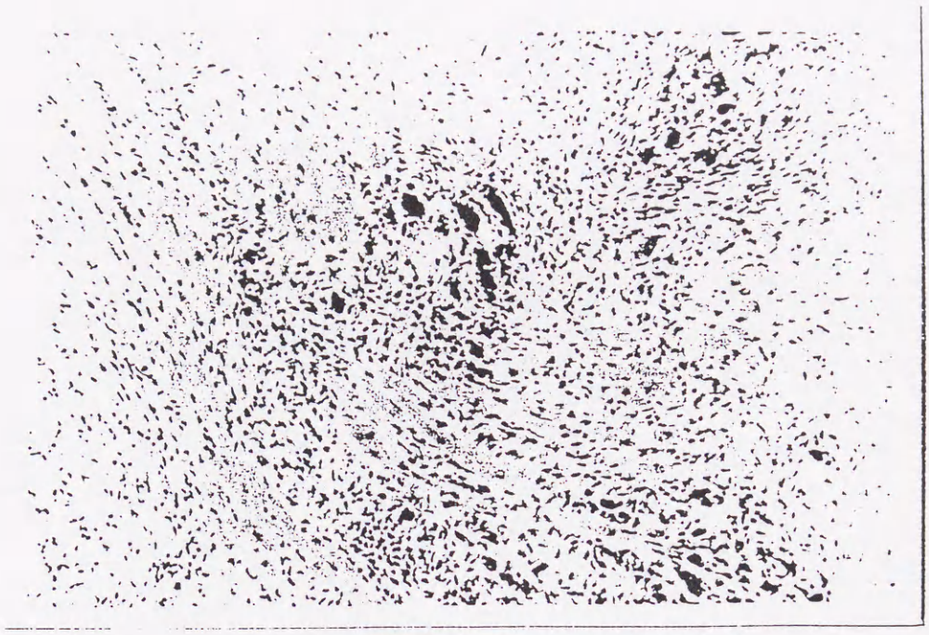
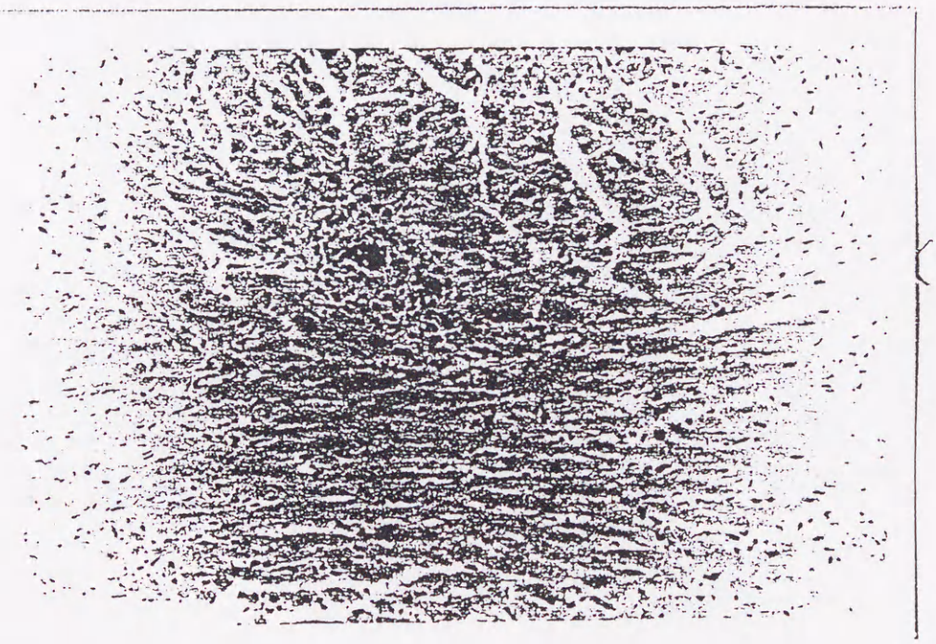

DAY 14
(Mn ±SD)
INFILTRATION
NECROSIS
CALCIFICATION
* P<0.05 VS CONTROL
** P<0.01 VS CONTROL
PATHOLOGIC GRADE
5+
4+
3+
2+
1+
**
**
*
**
**
*
CONTROL
PEG-SOD
1 ×10³
PEG-SOD
1 ×10³
(U/kg/day)
PEG-catalase
1 ×10³
(U/kg/day)
(n=18)
(n=10)
(n=14)

Figure 5

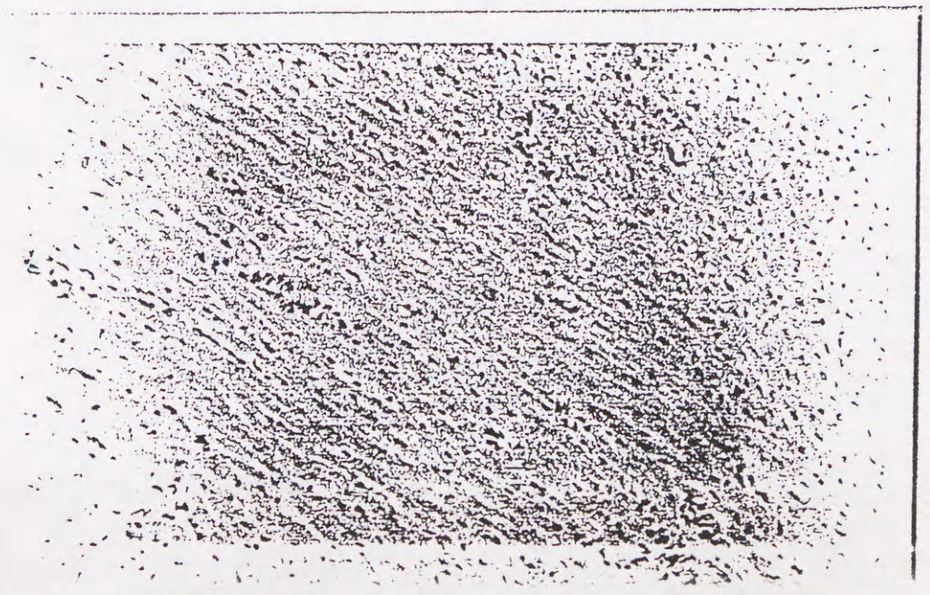
(A)

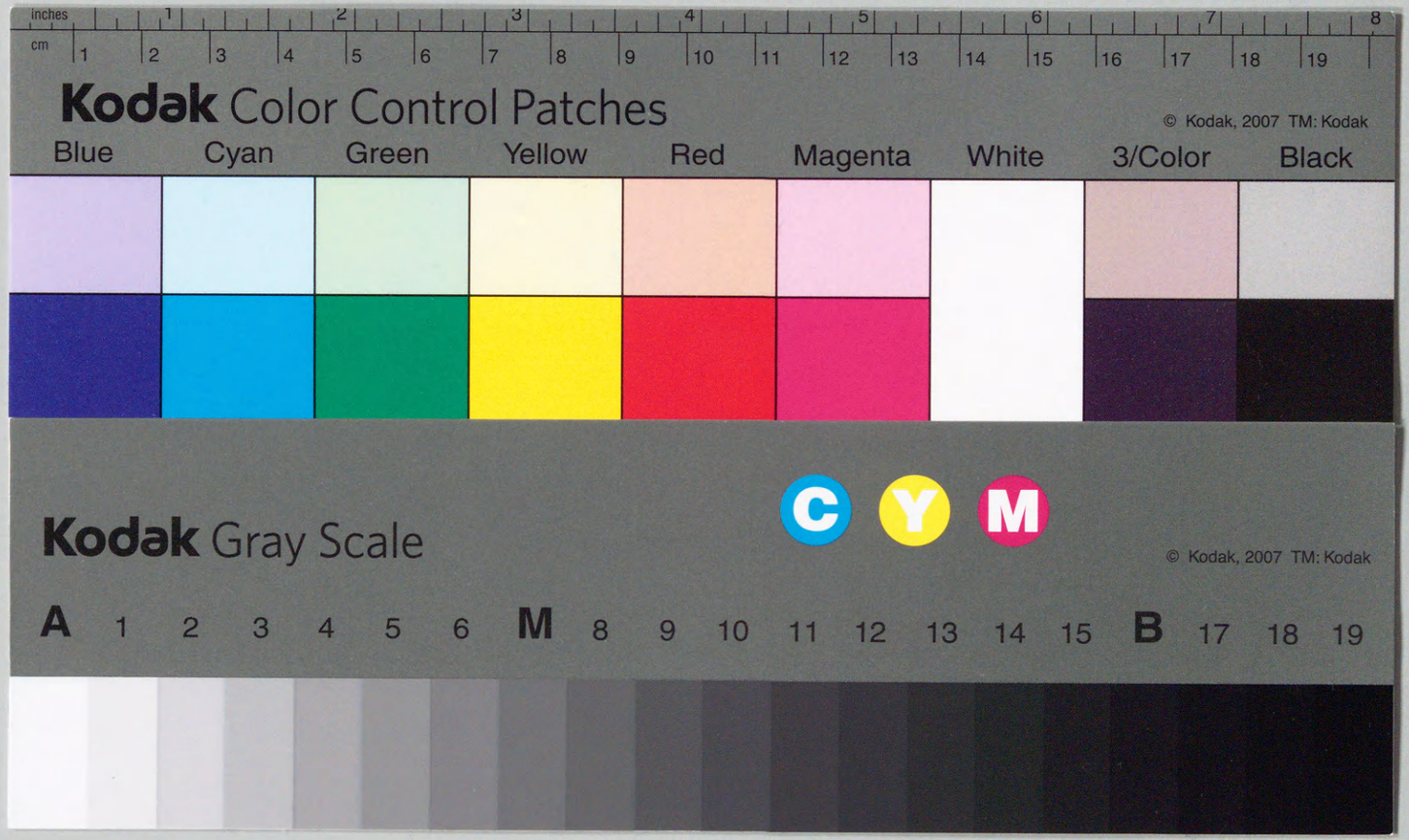


(B)



(C)




inches
cm
Kodak Color Control Patches
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Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19