Effect of EM-X and BF on Free Radicals *in vitro*

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Abstract
Objective: To study the effect of EM and BF on free radicals in vitro and to explore the anti-oxidative mechanisms of EM and BF.

Methods: According to the Wolf method that was slightly improved by us, homogenate of mitochondria of the rats' spleen was prepared. It was induced by Fe^{2+}-Vitamin C *in vitro* and cultivated at 37oC for 40 min. then the activities of CuNz-SOD, Mn-SOD, T-SOD, GSH-Px and CAT and contents of MDA, GSH, and T-AOC were measured, and compared with Vitamin C.

Results: EM-X and BF significantly inhibited the production of MDA, and improved the activity of antioxidative enzymes (T-SOD, CuZn-SOD, Mn-SOD, CAT and GSH-Px), and the content antioxidative substances in the cell such as GSH. They enhanced the total antioxidant competence (T-AOC). Effects of EM-X and BF are much stronger than that of Vitamin C.

Key words: EM-X, BF, splenic mitochondria, CuZn-SOD, Mn-SOD, T-SOD, GSH-Px, CAT, MDA, GSH, T-AOC

This experiment, as one of a series of studies of antioxidation for EM-X, was made in the lipid peroxidation of rats' spleen. Mitochondira of the spleen of rats was isolated and induced by Fe^{2+}-Vitamin C *in vitro*. The activities of copper zinc-superoxide dismutase (CuZn-SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) and contents of malondialdehyde (MDA), glutathione (GSH) and total antioxidant competence (T-AOC) were measure, and compared with Vitamin C. the results are shown as follows:

Methods

According to the Wolf method that was slightly improved by us, the rats were killed by decapitation, and the spleens were removed. The residual blood on the spleens was washed away and dried with filter paper. 10% homogenate of the spleens that had been weighted and cut into pieces with scissors was prepared with the homogenate instrument. The prepared homogenate was centrifugated at the rotating speed of 500rpm for 10 min and its deposit was cleared away. The up liquid was centrifugated at the rotating speed of 4000rpm for 10min and the deposit was removed away. The up liquid was centrifugated at rotating speed of 8500rpm for 10min at 4°C and then the up liquid was eliminated. Normal saline

solution of 4°Cwas added into the deposit, uniformly mixed. The mixture was centrifugated at the same condition to the last. The deposit was prepared into homogenate (mitochondria), which was further smashed in supersound disintegrator with 400A. This was repeated 3 times for 5 seconds with 10sec intervals between repetitions.

The experiment was divided into 7 groups: A: (Normal control, H_2O 0.1ml/tube), B: (Model control, H_2O 0.1ml/tube), C: (Vitamin 0.1ml/tube), and D: (Vitamin C 40°C 0.1 ml/tube), E: (EM-X 0.1ml/tube), F: (EM-X 40°C 0.1ml/tube) and G: (BF 0.1 ml/tube), and 4 test tubes were provided for each group. Treated as follows: all test tubes were first filled with 0.1 ml of corresponding drug fluid and then with 1ml of homogenate of mitochondria respectively (distilled water was added into Normal control and Model control test tube respectively to replace the drug fluid). 50μmol/l of $FeSO_4$ and 50μmol/l of Vitamin C were added into each test tube (distilled water was added into Normal control test tube to replace the drug fluid) after cultivating for 10 minutes at 37°C. Related data were measured according to given methods of the used test kits after cultivation for 30 minutes at 37°C.

Results

1. The effect on the content of MDA in splenic mitochondria (see Table 1):

Spontaneous MDA occurred in the rats' splenic mitochondria and the production of MDA was clearly increased ($P<0.01$) by induction of Fe^{2+}- Vitamin C. EM-X, EM-X heated, BF, and Vitamin C all obviously inhibited the production of MDA ($P<0.01$ or $P<0.05$). There were no differences in the inhibition of ME-X and heated EM-X and BF ($P<0.05$) in contrast to Vitamin C ($P<0.05$). The results are shown in Table 1 as follows:

Table 1 The Effect of EM-X, BF and Vitamin C on MDA Content

	Group	mean ± sd (MDA mmol/mg)
A	Normal Control	1.63 ± 0.86
B	Model Control	6.84 ± 1.05? ?
C	Vitamin C	4.40 ± 1.02? ? ??
D	Vitamin C of 40°C	6.17 ± 1.26? ? *
E	EM-X	2.03 ± 0.78??
F	EM-X of 40°C	2.51 ± 1.18??
G	BF	2.49 ± 1.03??

Compare with Group Normal control ? P<0.05, ? ? P<0.01. with Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

2. The effect on the content of GSH (see Table 2)

The content of GSH in the rats' splenetic mitochondira was clearly decreased ($P<0.01$). EM-X, EM-X heated, BF, and Vitamin C all obviously inhibited the decrease ($P<0.01$ or $P<0.05$), and the inhibition of EM-X and BF was much greater than that of Vitamin C ($P<0.01$ or $P<0.05$), but there were no differences

between heated and non-heated EM-X (P>0.05) in contrast to Vitamin C (P<0.05). The results are shown in Table 2 as follows:

Table 2 The Effect of EM-X, BF and Vitamin C on GSH Content

	Group	mean ± sd (GSH mmol/mg)
A	Normal Control	66.45 ± 14.46
B	Model Control	26.68 ± 14.21? ?
C	Vitamin C	46.33 ± 8.14? ?
D	Vitamin C of 40°C	30.42 ± 5.59? ? *
E	EM-X	75.81 ± 11.69? ? ** **
F	EM-X of 40°C	64.58 ± 6.92? ? ***
G	BF	66.36 ± 7.21? ? ***

Compare with Group Normal control ? P<0.05, ? ? P<0.01. with Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

3. The effect on T-AOC (see Table 3)

The T-AOC in the rats' splenic mitochondria was significantly decreased (P<0.01). EM-X, EM-X heated, BF, and Vitamin C all obviously inhibited the decrease (P<0.01), and the role of inhibition of EM-X and BF was much greater than that of Vitamin C (P<0.01), but the differences among heated and non-heated EM-X was not found (P<0.05) in contrast to Vitamin C (P<0.01). the results are shown in Table 3 as follows:

Table 3 The Effect of EM-X, BF and Vitamin C on T-AOC

	Group	mean ± sd (U/mg)
A	Normal Control	16.61 ± 2.37
B	Model Control	6.02 ± 1.70? ?
C	Vitamin C	11.59 ± 1.90? ?
D	Vitamin C of 40°C	7.65 ± 1.66? ? *
E	EM-X	18.07 ± 2.49? ? ** **
F	EM-X of 40°C	17.61 ± 2.48? ? ***
G	BF	17.55 ± 2.35? ? ***

Compare with Group Normal control ? P<0.05, ? ? P<0.01. With Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

4. The effect on the activity of CAT (see Table 4)

The activity of CAT in the rats' splenic mitochondria was markedly decreased (P<0.01). EM-X, EM-X heated, BF, and Vitamin C all significantly inhibited the decrease (P<0.01), and the action of inhibition of EM-X and BF was much greater than that of Vitamin C (P<0.01), but no differences among heated and non-heated EM-X appeared (P>0.05) in contrast of Vitamin C (P<0.01). The results are shown in Table 4 as follows:

Table 4 The Effect of EM-X, BF and Vitamin C on CAT Activity

	Group	mean ± sd (U/mg)
A	Normal Control	13.11 ± 1.78
B	Model Control	3.19 ± 2.11? ?
C	Vitamin C	8.59 ± 1.99? ??
D	Vitamin C of 40°C	3.53 ± 2.72? ? **
E	EM-X	17.19 ± 4.62??****
F	EM-X of 40°C	16.63 ± 1.46??****
G	BF	16.29 ± 1.98??****

Compare with Group Normal control ? P<0.05, ? ? P<0.01. With Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

5. The effect on the activity of GSH-Px (see Table 5)

The activity of GSH-Px in the rats' splenic mitochondria was slightly improved (P<0.05). EM-X, EM-X heated, BF, and Vitamin C all further increased the activity GSH-Px (P<0.01), and the improving effect of EM-X and BF on the activity was much greater than that of Vitamin C (P<0.01), but there were no differences among heated and non-heated EM-X (P<0.05) in contrast to Vitamin C (P<0.01). The results are shown in Table 5 as follows:

Table 5 The Effect of EM-X, BF and Vitamin C on GSH-Px Activity

	Group	mean ± sd (U/mg)
A	Normal Control	6.24 ± 1.89
B	Model Control	8.45 ± 1.89
C	Vitamin C	13.03 ± 1.72? ? ??
D	Vitamin C of 40°C	8.87 ± 2.17**
E	EM-X	19.96 ± 2.60? ? ??****
F	EM-X of 40°C	18.29 ± 1.43? ? ??****
G	BF	18.37 ± 1.65? ? ??****

Compare with Group Normal control ? P<0.05, ? ? P<0.01. With Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

6. The effect on the activity of T-SOD (see Table 6)

The activity of T-SOD in the rats' splenic mitochondria was clearly decreaed (P<0.01). EM-X, EM-X heated, BF, and Vitamin Call obviously reduced the decrease (P<0.01), and the reducing action of the decrease of EM-X and BF was much greater than that of Vitamin C (P<0.01) without differences between heated and non-heated EM-x (P<0.05) in contrast to Vitamin C (P<0.01). The results are shown in Table 6 as follows:

Table 6 The Effect of EM-X, BF and Vitamin C on T-SOD Activity

	Group	mean ± sd (NU/mg)
A	Normal Control	66.10 ± 1.12
B	Model Control	27.98 ± 1.96? ?
C	Vitamin C	59.79 ± 1.28? ? ??

D	Vitamin C of 40°C	28.65 ± 0.55? ? **
E	EM-X	76.36 ± 0.63? ? ??****
F	EM-X of 40°C	74.68 ± 1.17? ? ??****
G	BF	74.31 ± 1.15? ? ??****

Compare with Group Normal control ? P<0.05, ? ? P<0.01. With Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

7. The effect of CuZn-SOD activity (see Table 7)

The activity of CuZn-SOD in the rats' splenic mitochondria was lowered greatly (P<0.01). EM-X, EM-X heated, BF and Vitamin C all significantly eased the decrease (P<0.01), which of EM-X and BF was much greater than that of Vitamin C (P<0.01), but there were no differences in effect of heated and non-heated EM-X on easing the decrease (P<0.05) in contrast to Vitamin C (P<0.01). The results are shown in Table 7 as follows:

Table 7 The Effect of EM-X, BF and Vitamin C on CuZn-SOD Activity

	Group	mean ± sd (NU/mg)
A	Normal Control	29.64 ± 0.84
B	Model Control	11.25 ± 1.22? ?
C	Vitamin C	27.12 ± 0.82? ? ??
D	Vitamin C of 40°C	11.31 ± 1.33? ? **
E	EM-X	33.72 ± 1.62? ? ??****
F	EM-X of 40°C	32.22 ± 1.81? ? ??****
G	BF	32.20 ± 1.17? ? ??****

Compare with Group Normal control ? P<0.05, ? ? P<0.01. With Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

8. The effect on Mn-SOD activity (see Table 8)

The activity of Mn-SOD in the rats' splenic mitochondria clearly was lowered (P<0.01). EM-X, EM-X heated, BF, and Vitamin C all obviously eased the decrease (P<0.01). Whiles the role of EM-X and BF was much greater than that of Vitamin C (P<0.01), non-heated EM-X was no difference (P>0.05). the results are shown in Table 8 as follows:

Table 8 The Effect of EM-X, BF and Vitamin C on Mn-SOD Activity

	Group	mean ± sd (NU/mg)
A	Normal Control	36.46 ± 0.48
B	Model Control	16.73 ± 1.96? ?
C	Vitamin C	32.67 ± 0.95? ? ??
D	Vitamin C of 40°C	17.34 ± 1.84? ? **
E	EM-X	42.64 ± 1.52? ? ??****
F	EM-X of 40°C	42.65 ± 2.41? ? ??****
G	BF	42.41 ± 1.85? ? ??****

Compare with Group Normal control ? P<0.05, ? ? P<0.01. With Group Model control ?P<0.05, ??P<0.01, with Group Vitamin C*P<0.05, **P<0.01 and with Group Vitamin C of 40°C*P<0.05, **P<0.01

Discussion

Researchers in recent years indicate that cancers, radio-injuries and aging occurrence and development of many diseases are closely related to free radical injuries. The key reason is that free radicals from organs injure biomembranes, enzymes and nucleic acid, etc. and result in a series of injury processes such as producing lipid peroxides (LPO). However, there are natural systems of protection against free radicals, such as antioxidant system: Vitamin E, Vitamin C and GSH and so on, and antioxidase system: SOD, GSH-Px and CAT, etc. Which are able to clear free radicals awayto protect body.

Antioxidative mechanism of EM-X and BF was commonly explored in this study and the results reveal that they exert strong effect on anti-oxidative system in the main organs or rats. Therefore, we can conclude that the action against injuries of free radicals for EM-X and BF probably result from the protection and improvement of activity of antioxidase (CuZn-SOD, Mn-SOD, GSH-Px, and CAT) system and reducing the production of lipid peroxides.

Conclusions

The experiment results indicate that EM-X and BF exert strong anti-oxidant competence in the main organs of rats such as spleen mitochondria, which are shown in the following respects:

1. Inhibiting the production of MDA.
2. Improving the activity of anti-oxidative enzymes (T-SOD, CuZn-SOD, Mn-SOD and GSH-Px).
3. Increasing the content anti-oxidative substances in the cell such as GSH, further enhancing the total anti-oxidant competence (T-AOC).
4. Effects of EM-X and BF are much stronger than that of Vitamin C.

Methodology

1. Materials and animals

a. EM-X: EM Research Organization, Inc., Japan, licensed number: OB0005264121
b. BF (Bifidotein, product created through fermentation with bifidobacterium): Instituteee of Medical & Pharmaceutical Science Qiqihar, P. R. China, licensed number: 20010711
c. Vitamin C: 12.5g/100ml Chemical Test Material Whole-sale Station, Harbin Medicine Company, licensed number: 1999413
d. Malondialdehyde (MDA) Test Kits: Nanjing Jiancheng Bio-engineering Research Institute, licensed number: 20010313
e. Glutathione (GSH) Test Kits: Nanjing Jiancheng Bio-engineering Research Institute, licensed number: 20010308
f. Total antioxidant competence (T-AOC) Test Kits: Nanjing Jiancheng Bio-engineering Research Institute, licensed number: 20010313
g. Catalase (CAT) Test Kits: Nanjing Jiancheng Bio-engineering Research Institute, licensed number: 20010314

h. Glutathione peroxidase (GSH-Px) Test Kits: Nanjing Jiancheng Bio-engineering Research Institute, licensed number: 20010313
i. Superoxide dismutase (SOD) Test Kits: Nanjing Jiancheng Bio-engineering Research Institute, licensed number: 20010308
j. Wister rats were provided by Animal Experiment Center, Qiqihar Medical College.