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Rodent Macrophage Select Vin Blank Together with Vin Rouge According to SO Level *in Situ*

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Abstract

Phagocytic cells are known as multi potent activities for its ontogenical and phylogenetical aspect. One of the strike actions is super oxide anion known as critical role of the cell when microorganisms invade inside the cytoplasm. This agent sometimes triggered as a serious factor in the site of inflammation. There are many papers which concerned newly accessed anti-oxidative substances. However, many reports only focused on the molecular basis *in vitro*, suggesting vin rouge for their rich content of polyphonol rather than vin blank with a French paradox. We had been suggested that the key factor for oxidative stress needs to be discussed first for immunological standpoint, such as in phagocytic cell *in situ*. With this assay system in rodent macrophage, SO level was about the same for both groups administered vin blank as well as vin rouge.

Keywords

Super Oxide Anion, Anti-Oxidant Sample, Neutrophyl, Macrophage, Vin Blank, Vin Rouge, *In Situ* Estimation

1. Introduction

A phagocytic cell is famous for its mobile activity as the scavenger in the front of innate and adoptive immune

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system in mammals [1]-[5]. In a lower animal, even in invertebrate one, the phagocytic cell is essential for surviving a conventional circumstance [6]-[10]. In such cell, super oxide anion had been known as critical role in a phagocytic cell when microorganisms invaded inside [11]-[14]. But, this anion is not always necessary in a clean environment such as in the developed country. Moreover, this agent sometimes triggered as serious factor in the site of inflammation. There are many papers which concerned newly found anti-oxidants [15]-[26]. However, many reports only discussed about the molecular bases of mechanism *in vitro*/in test tube, and how oxidative stress damaged the human tissue. The key factor for oxidative stress has to be discused first for immunological standpoint, and with so-called armed reduction treaty. In other words, a minimum armed force is necessary for an invasion by outer or inner irregular cells. Many reports already reported that vin rouge reveals anti-oxidative effect but not vin blank. For the above standpoint on the *ex vivo* system by phagocytic cell, it is necessary to review the anti-oxidative effect of wine. So we tried to estimate anti-oxidative activity directory by employing peritoneal murine macrophage. The purpose of this study is to establish a conventional method which is useful for evaluating the antioxidant activity directory with host cell, macrophage. Oxidative stress is an important process that occurs *in vivo* during aging and is considered one of the main causes of molecular damage to cellular and tissue structures. These changes can gradually accumulate in the original biological structures during aging.

With aging, there is an increase of oxidative stress due to an imbalance between the oxidant production and the antioxidant levels. Since immunological cell functions are specially linked to free radical generation, the oxidant and antioxidant balance is essential for these cells. Although low levels of anti-oxidants bring armless status of a decreased in immune system, high levels of anti-oxidants could damage the tissues [27]-[34]. So ideal system was a regulation of daily status of immunological condition by phagocyte.

2. Subjects and Methods

2.1. Animals

Eight weeks old female C57BL/6NCrj were purchased from Sankyo Laboratory Service Corporation (Shizuoka, Japan). All mice were kept under SPF: specific pathogen-free conditions. The water and food were freely provided for each animal.

2.2. Preparation of Peritoneal Macrophage

Subjects

As for the basic medium, HEPES buffer (HEPES 17 mM, NaCl 120 mM, Glucose 5 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM) was prepared and sterilized by filtration. Phrbol 12-myristate 13-acetate (PMA, Sigma, USA) was diluted to 10 - 6 M by dimethylsulfoxide (DMSO, Sigma, USA) and used as a stimulant for super oxide anion generation of murine peritoneal exudative cells. Cytochrome-C (Sigma, USA) was diluted to 1 mM by HEPES buffer. Since cytochrome-C reduced by super oxide showed maximum absorbance at 550 nm, we used cytochrome-C to measure the amount of super oxide anion generation through spectrophotometrical technique. Oyster Glycogen (type II, Sigma, USA) was diluted in the purified water (10% w/v, Wako, Japan) and autoclaved at 120˚C for 20 min. This solution was used for intraperitoneal injection to mice in order to induce peripheral neutrophils into the abdominal cavit.

In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), famous enzyme for its anti-oxidative effect, into our experimental system. The result was as expected that the reduction of cytochrome-C was inhibited just after addition of SOD. This showed us that our experimental system could be used properly for measuring the amount of generated super oxide anion.

2.3. Reagents

As for the basic medium, HEPES buffer (HEPES 17 mM, NaCl 120 mM, Glucose 5 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM) was prepared and sterilized by filtration. Phorbol 12-myristate 13-acetate (PMA, Sigma, USA) was diluted to 10^{-6} M by dimethyl sulfoxide (DMSO, Sigma, USA) and used as a stimulant for super oxide anion generation of murine peritoneal exudative cells. Cytochrome-C; Cyt-C, (Sigma, USA) was diluted to 1 mM by HEPES buffer. Since Cyt-C reduced by super oxide showed maximum absorbance at 550 nm, we

used Cyt-C to measure the amount of super oxide anion generation through spectrophotometrical technique.

Oyster Glycogen (type II, Sigma, USA) was diluted in the purified water (10% w/v, Wako, Japan) and autoclaved at 120˚C for 20 min. This solution was used for intraperitoneal injection to mice in order to induce peripheral neutrophils into the abdominal cavity.

2.4. Anti-Oxidative Sample

Commercially available red and white wines were prepared and then aspirated overnight at ice cold bath in order to eliminate alcohol for mice. Followings are the commercially available wine for serve this experiment.

For Vin Blanc, "Château Cantemerle 1996 Haut Médoc 5ème Grand Cru Classé" was selected at Japanese wine delivery shop.

For Vin ouge, "Haut-Médoc Cru Bourgeois 2008 Château Bel Air" was selected as the same class of price at the delivery.

2.5. A measuring the Amount of Super Oxide Anion Generated by Murine Peritoneal Macrophage Administered Vin Blanc or Vin Rouge

Each wine sample was orally administered to mice (200 mg/kg) every day for one week. Then two ml of 10% oyster glycogen was injected intraperitonealy about 10 hours before the assay. A sufficient murine peritoneal exudate cells (PEC) were induced ten hours after the stimulation. Mice were euthanized by cervical dislocation, murine PEC suspension was centrifuged twice for 5 minutes at 1500 rpm, 4˚C. Then PEC was prepared to 1×10^6 cells/ml of HEPES buffer. One hundred µl cytochrome-C and 10 µl of PMA were added to the cell suspension and this was incubated for 20 minutes at 37˚C. The reaction mixture was then centrifuged for 10 minutes at 1500 rpm, 4˚C. The OD of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was shown in the formula; increased absorbance at 550 nm $(\Delta A_{550\text{-}540})/19.1 \times 10^3$ mmol/ml.

In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), famous enzyme for its anti-oxidative effect [21]-[23], into our experimental system. The result was as expected that the reduction of cytochrome-C was inhibited just after addition of SOD. This showed us that our experimental system could be used properly for measuring the amount of generated super oxide anion.

2.6. Statistical Analysis

The statistical comparisons between the groups for the test of significant difference were performed using paired t-test and Wilcoxon signed-ranks test. Further, the test of the correlation were performed a spearman's correlation coefficient by rank test. Data are expressed as means ± standard error of mean (SE). A P value < 0.05 was considered to be statistically significant.

Data are expressed as means ± standard deviations. The differences between HF-treated and non-treated conditions were compared using a one-tailed analysis of variance. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. The Amount of Generated Super Oxide Anion and Chasing with SOD

The peritoneal excaudate cells were collected 5 days after injection with oyster glycogen. The cell sample consisted with 92% of macrophages. The collected cell was treated by the method described in the Section 2.5. Before starting the main topics of this report, we try to check this reaction in **Figure 1** depend on a super oxide anion. To confirm this SOD solution were mixed with the main cell suspension after concecutive minutes, post starting the reaction (**Figure 1**).

The amount of generated super oxide anion was calculated in the formula shown in the Section 2.5 (**Table 1**).

Figure 2, sufficient murine peritoneal exudate cells were induced ten hours after the stimulation. Mice were euthanized by cervical dislocation, murine peritoneal exudate cells (PEC) suspension was centrifuged twice for 5 minutes at 1500 rpm, 4˚C. Then PEC was prepared to 1×10^6 cells/ml of HEPES buffer. One hundred µl cytochrome-C and 10 µl of PMA were added to the cell suspension and this was incubated for 20 minutes at 37˚C.

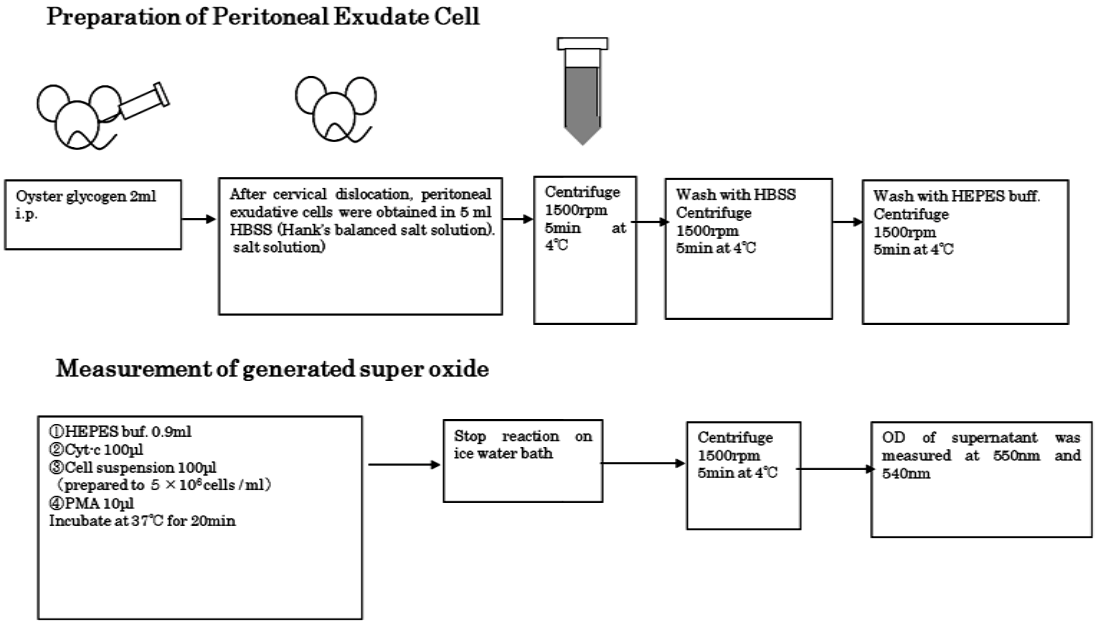

Figure 1. Experimental Design from *Open Journal of Rheumatology and Autoimmune Diseases*, 2014, 4, 13-21 with permission. A sufficient murine peritoneal exudate cell (PEC) was induced ten hours after the stimulation. Mice were euthanized by cervical dislocation, then murine PEC suspension was centrifuged twice for 5 minutes at 1500 rpm, 4˚C. Then PEC was prepared to 1×10^6 cells/ml of HEPES buffer. One hundred μl cytochrome-C and 10 μl of PMA were added to the cell suspension and this was incubated for 20 minutes at 37˚C. The reaction mixture was then centrifuged for 10 minutes at 1500 rpm, 4˚C. The OD of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was shown in the formula; increased absorbance at 550 nm $(\Delta A_{550\text{-}540})/19.1 \times 10^3$ mmol/ml. In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), famous enzyme for its anti-oxidative effect, into our experimental system. The result was as expected that the reduction of cytochrome-C was inhibited just after addition of SOD. This showed us that our experimental system could be used properly for measuring the amount of generated super oxide anion.

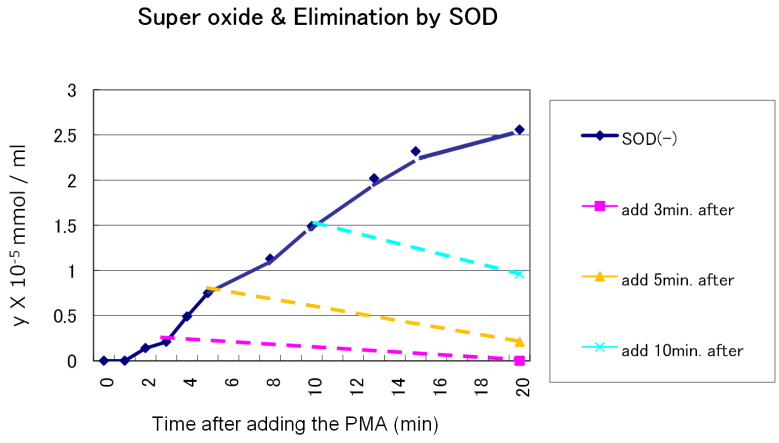

Figure 2. Chasing of super oxide activity by SOD. SOD was diluted to 1mg/ml by HEPES buffer. Thirty μl of SOD was added to the reaction mixuture 3, 5 and 10 minutes after incubation, the reduction of cytochrome-C was inhibited just after adding of SOD. Then PEC was prepared to 1×10^6 cells/ml of HEPES buffer. One hundred μl cytochrome-C and 10 μl of PMA were added to the cell suspension and this was incubated for 20 minutes at 37˚C. The reaction mixture was then centrifuged for 10 minutes at 1500 rpm, 4˚C. The OD of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was shown in the formula; increased absorbance at 550 nm $(\Delta A_{550\text{-}540})/19.1 \times 10^3$ mmol/ml. In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), famous enzyme for its anti-oxidative effect, into our experimental system after 3, 5 and 10 minutes after the reaction.

Table 1. In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), famous enzyme for its anti-oxidative effect, into our experimental system. The reaction was clearly chased by mixing SOD.

Group	Generated O_2	
	Before Chasing	After Chasing
3 Minutes	0.29	0.02
5 Minutes	0.81	0.12
10 Minutes	1.52	0.95
20 Minutes	2.58	-

The reaction mixture was then centrifuged for 10 minutes at 1500 rpm, 4˚C. The OD of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was shown in the formula; increased absorbance at 550 nm $(\Delta A_{550\text{-}540})/19.1 \times 10^3$ mmol/ml.

In order to ensure if we really measured the amount of generated super oxide anion or not, we tried to add super oxide anion dismutase (SOD), famous enzyme for its anti-oxidative effect, into our experimental system.

3.2. The Comparison of Generated Super Oxide Anion between Vin Blank and Vin Rouge

Three groups of mice were set up by each ten individuals. Each mouse was orally administered 0.5 ml of alcohol-less wine.

The experimental animals were orally administered by each sample for 200 mg/kg by a stomach tube. The peritoneal excaudate cells were collected 5 days after injection with oyster glycogen. The cell sample consisted with 92% of macrophages. The collected cells were treated by the method described in the Section 2.5. The amount of generated super oxide anion was calculated in the formula shown in the Section 2.5 (**Table 2**).

Both groups of mice that administered each wine were responding to antioxidative response. However, there was no difference between the group administered vin rouge and vin blank. Rather vin blank group was tend to more strong anti-oxidative response, but there is no significant difference in both wine group.

Figure 3, three groups of mice were set up by each ten individuals. Each mouse was orally administered 0.5 ml of alcohol-less wine.

The experimental animals were orally administered by each sample for 200 mg/kg by a stomach tube. The peritoneal excaudate cell was collected 5 days after injection with oyster glycogen. The cell sample consisted with 92% of macrophages. The collected cell was treated by the method described in the Section 2.5. The amount of generated super oxide anion was calculated in the formula shown in the Section 2.5.

4. Discussion

Reactive oxygen species released by activated polymorphonuclear leukocyte as an expression of their definitive function are considered to be major source of cytotoxic oxidative stress. Since oxygen free radicals cause DNA damage and this may lead to gene modifications, reactive oxygen might be carcinogenic. Therefore oxidative stress is reported to cause many disorders such as cancer, ischemic heart disease and diabetes [27]-[29].

However, like armed forces reduction treaty, the minimum armed forces are necessary for our environment except germ free environment. Although there are many reports in which the newly found antioxidants were reported and discussed about the molecular mechanism of how oxidative stress damage the human tissue [30]-[34], few experiments have been done *in vivo* which could reflex more precisely the biological response.

The purpose of this study has been to establish the method which is useful for evaluating the activity of antioxidant *ex vivo*. As shown above, in our experiment design, the amount of super oxide anion generation decreased significantly after one-week administration of some drugs. The advantage of this method might be what the amount of super oxide anion generation could express clearly in concrete numeral, so it is easy to compare various drugs.

The most interesting in this assay was that the generation of super oxide anion was much more inhibited after

Table 2. The experimental animals were orally administered by each sample for 200mg/kg by a stomach tube. The peritoneal excaudate cells were collected 5 days after injection with oyster glycogen. The cell sample consisted with 92% of macrophages. The collected cells were treated by the method described in the Section 2.5. The amount of generated super oxide anion was calculated in the formula shown in the Section 2.5. Both group of mice that administered each wine was respond to anti-oxidative response . However, there was no difference between the group administered vin rouge and vin blank. Rather vin blank group was tend to more strong anti-oxidative response, but there is no significant difference in both wine group. The real value of figure was shown in the Table.

Group	Generated O_2
Control	3.52 ± 0.43
Vin Blanc	2.25 ± 0.35***
Vin Rouge	2.39 ± 0.56***

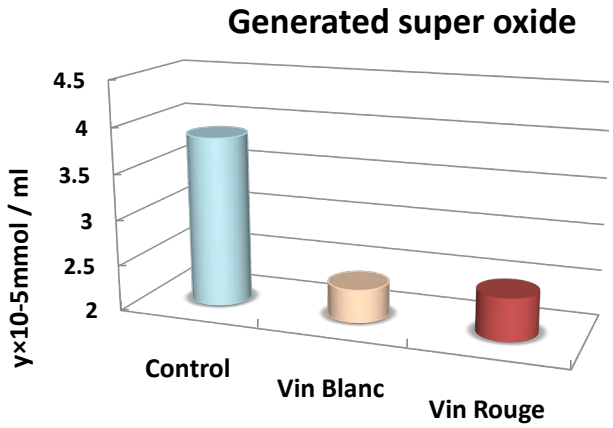

Figure 3. A judging anti-oxidative activity by the macrophages from both groups. Each wine sample was orally administered to mice (200 mg/kg) every day for one week. Then two ml of 10% oyster glycogen was injected intraperitonealy about 10 hours before the assay. A sufficient murine peritoneal exudate cell was induced ten hours after the stimulation. Mice were euthanized by cervical dislocation, murine PEC suspension was centrifuged twice for 5 minutes at 1500 rpm, 4˚C. Then PEC was prepared to 1×10^6 cells/ml of HEPES buffer. One hundred µl cytochrome-C and 10 µl of PMA were added to the cell suspension and this was incubated for 20 minutes at 37˚C. The reaction mixture was then centrifuged for 10 minutes at 1500 rpm, 4˚C. The OD of supernatant was measured at both 550 nm and 540 nm, the amount of generated super oxide anion was shown in the formula; increased absorbance at 550 nm $(\Delta A_{550\text{-}540})/19.1 \times 10^3$ mmol/ml.

white wine administration than red wine. It is widely accepted that polyphenol contained in red wine has great anti-oxidative effects. However, as we demonstrated in this report, white wine has more efficient anti-oxidative activity. The confusion that red wine prefers anti-oxidative effects was pulled out by the results from *in vitro* system. We would like to stress the anti-oxidative test should first be evaluated by the *in vivo* system employing the cells work at the front of battle line of defense by phagocytic cell. The phagocytic cells have much oxidative enzyme against foreign microorganisms. Moreover this confusion biased from the colored substance such as anthocyanin combined directory with the color of red wine. Thus, we would like to recommend testing the anti-oxidative activity, if possible, *in vivo* in future.

In this report, the method how to measure the amount of super oxide anion generation when both wines were administered *in vivo* was shown. Using this method, it would be easy to test the anti-oxidative activity of any drugs and supplements for human subject. This will bring the difference of oxidative activity in monocyte in human blood, as qualitative aspect. The anti-oxidative stress should be regulated as armed forces reduction treaty, but not dissemination of armed forces [35]-[39].

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