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TBA Reactive Substance in Experimental Brain Edema

Michio Yamaguchi

Malonic dialdehyde (MDA) is considered to be produced by lipoperoxidation in the tissue. Since MDA reacts with thio-barbituric acid (TBA) and gives a red colored substance, the estimation of MDA has been carried out widely by TBA reaction method. However, the reproducibility of this method was not satisfactory. The aim of this study is to determine the usefulness of TBA reaction method for the detection of MDA in the experimental brain edema of rats.
A strictly narrow temperature zone was not required for the optimal color developing reaction. When the tissue homogenate contained a kind of antioxidants, i.e. butylated hydroxytoluene, and when the tissue was immediately frozen by liquid nitrogen after decapitation, the spontaneous increase of MDA was minimal. If the concentration of the brain homogenate was over 5%, the MDA also increased spontaneously in the test tube during reaction steps.
Anti-brain edema efffect of a free radical scavenger, N,N'propylene bisnicotinamide (AVS) was evaluated by the estimation of tissue water content, specific gravity, beta-glucuronidase activity and MDA content on a model of experimental brain edema. Although significant protection on the increase of MDA was not observed, other parmeters showed anti-edema effect by AVS administration.

Key Words
Malonic dialdehyde,
Brain edema,
Thio-barbituric acid,
Concentration of homogenate,
Free radical scavenger.

INTRODUCTION

Since brain edema is a condition caused by the disturbance of the membrane function, free radicals may play some important role on the initiation and on the development of this pathological process in the brain. Polyunsaturated lipid of the membrane are believed to be the target on the oxidative disintegration. Malonic dialdehyde (MDA) is considered to be produced as the result of the lipoperoxide formation. MDA reactive substance of red color is produced by the reaction of MDA with thio-barbituric acid (TBA) in tubes at around 90°C. However, the determination of the TBA reactive substance in the tissue preparation is controversial because of poor reproducibility on the assay process. The content of TBA reactive substance, therefore, may not reflect the rate of the severity of the brain edema correctly. In this laboratory, some technical conditions were checked carefully on the color developmental process of TBA reactive substance and the reliability of this method will be discussed on the experimental brain edema preparation.

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MATERIALS AND METHODS

TBA reactive substanc was assayed by the method of Ohkawa et al. (1) The procedure was carried out in the following manner. MDA was reacted with thio-barbituric acid at pH 3.5 by acetic acid buffer. The reaction was maintaind for 30 min at 80, 90, or 97°C as specifid in Table 1. These reacting temperatures were adjusted and kept by the use of an aluminum heat lack. Tetramethoxypropane or tetraethoxypropane was used as a standard of MDA. The content of the red colored final substance was determined by Hitachi spectrophotometer, model 100–20 as absorbance at 532 nm.

Experimental brain edema was produced by the method of Levine and Torrelio (2) using Wistar strain male rats of 250–350 g. After the making a small burr hole on the skull, a small amount of silver nitrate was semi-stereotaxically introduced into the right parietal region under the pentobarbital anesthesia, Animals were, then, divided into two groups. One group received 100 mg/kg of N,N'-propylene bisnicotinamide (AVS), a free radical scavenger, intraperitoneally every 6 hour, and was called AVS group. The control group had 0.25 ml of normal saline instead of AVS. Twenty four hours later, the animal was sacrificed by decapitation. For the study of spontaneous increase of MDA, the head was immediately frozen by the immersion in liquid nitrogen. The frozen tissue was excised and weighed very quickly, and homogenized with ice-cold 0.9% saline which contained ethanol (1%) and butylated hydroxytoluene (BHT) as an antioxidant (0.1%). Ethanol was used for the solvent of BHT. In other instance, the freshly taken tissue from the skull within 60 seconds was homogenized with BHT containing solution, and MDA was assayed on the homogenate. The homogenization was performed by the use of Potter Elvehjem type homogenizer with motor driven teflon pestle. Clearance was approximately 0.10 to 0.15 mm. The stroke was 7 times of up and down procedures. All homogenizntion was performed in ice-cold water bath.

The specific gravity of the brain tissue was determined by the method of Nelson et al (3). Beta-Glucuronidase activity was determined by the method of Fishman et al (4) with modification. AVS was kindly supplied from Chugai Seiyaku, Ltd., Tokyo. All other chemi-

Table 1. Effect of temperature during the reaction with TBA.

℃	Extinction at 532 nm		
	10 nmol*	30 nmol	50 nmol
97	.33	1.18	o.s.
90	.33	.98	o.s.
80	.27	1.01	o.s.

* Tetraethoxypropane o.s. : over scaled

Table 2. Preparation of TBA reagent.

Solution	Extinction at 532 nm		
	10 nmol*	30 nmol	50 nmol
Freshly prepared	.127	.419	.732
7 days old	.139	.448	.828

* Tetraethoxypropane

Table 3. MDA (nmols/g wet tissue) in various homogenates.

Brain	Homogenizing solvent*	Incubation for 30 min.	
		at 0℃	at 38℃
Frozen	Saline	291.7±28.2	273.0±9.8
	BHT	178.5±6.1	173.0±10.6
Fresh	Saline	458.0±42.5	—
	BHT	206.5±1.2	—

*chilled

All data expressed as mean±SD (n=3).
Saline : contained ethanol (1 %).
BHT : contained ethanol (1 %) and BHT (0.1%).

cals were reagent grade.

RESULTS

1. Temperature at the Reaction and TBA Solution

Various temperature (80, 90, and 97°) for the reaction were tested and the result was shown in Table 1. If the temperature was over 80°, color development was considered to be almost same rate. TBA solution was also checked if the freshly prepared one was much more effective. But no difference was noted when compared with the 7 day old solution as shown in Table 2.

2. Tissue Preparation

In Table 3, the instantly frozen brain after decapitation and the non-frozen brain were compared on their content of MDA. Also, the effect of antioxidant in the homogenizing solution was checked by the following condition: the homogenizing solution contained 1% ethanol with or without 0.1% BHT. These homogenates were taken for the color developing reaction.

The frozen brain tissue homogenized with BHT containing solution gave the lowest value of MDA. After homogenization, no distinct increase of MDA was detected if the homogenate was kept for 30 minutes at either 0 or 38°C.

3. Concentration of Homogenate

Using various conceatration of homogenate, the MDA content was compared as shown in Figure 1. Fifty μl, 100 μl, 400 μl, and 800 μl of 20%, 10% 5%, 2.5%, and 1.25% of homogenate, respectively, were reacted with

TBA at pH 3.5. Since the content in the tissue (nmols/g) was correced in calculation, the similar amount of MDA should be theoretically estimated in each reaction test tube because the same brain sample was used. When the concentration was over 5%, however, the content of MDA increased steeply by the order of homogenate concentration. To prevent the spontaneous increase of MDA in the test tube during the reaction, the homogenate must contain 5% or less tissue even in the BHT containing condition.

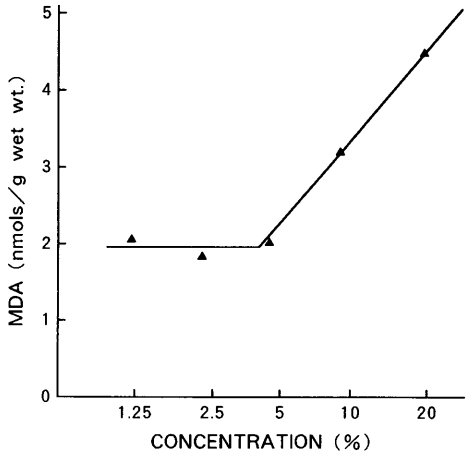

Figure 1. MDA in homogenates of various concentration.

4. Specific Gravity and MDA in the Edematous Tissue

As shown in Table 4, the specific gravity of the right side brain tissue in the saline group was significantly lower than that of left one ($P < 0.01$). When AVS was given, the specific gravity of the right parietal region was 1.0440 ± 0.0015 (mean ± S.D.). This value was significantly lower than that of untreated edema tissue (right side of saline group) and significantly higher than that of control tissue (left side of AVS group).

The content of MDA in the edematous tissue in the saline group was significantly increased when compared with that of control side ($P < 0.05$) as shown in Table 5. However, AVS did not give any significant improvement on the content of MDA.

5. Beta-Glucuronidase Activity of Brain Tissue

Although AVS did not prevent the increase of MDA in the edematous tissue, the increase of the lysosomal enzyme activity was prevented by AVS as shown in Table 6.

DISCUSSION

Although Suzuki and Yagi firstly reported that the MDA content increased in the cold induced brain edema tissue (5), Tanaka et al denied the change of

Table 4. Specific gravity of brain tissue.

Side	Saline Group	AVS Group
Right (BE)	1.0424±.0018(9)*	1.0440±.0015(12)**
Left (Control)	1.0468±.0005(9)***	1.0477±.0007(13)

*P<0.025 vs. BE Side of AVS.
**P<0.001 vs. Control Side of AVS.
***P<0.01 vs. BE Side of AVS.

Table 5. MDA (nmols/g) in brain tissue.

Side	Saline Group	AVS Group
Right (BE)	258.3±15.6(6)*	254.8±27.8(10)**
Left (Control)	224.0±16.9(5)	202.6±50.1(9)

*P<0.005 vs. Control Side of Saline group.
**P<0.01 vs. Control Side of AVS group and non significant vs. BE Side of Saline group.

Table 6. Water contents and beta-glucuronidase activities.

Group		Water Content (%)	β-Glucuronidase (μg PP/mg/h)
Saline	R	82.52±1.26 (14)	0.428±0.096 (14)
	L	80.00±0.80 (15)	0.146±0.071 (14)
AVS	R	80.59±0.67 (6)	0.166±0.049 (12)
	L	78.41±0.46 (6)	0.167±0.059 (12)
Normal Control		79.00±0.29 (8)	0.200±0.042 (10)

MDA content in the edematous or ischemic brain tissues (6). Those workers did not use antioxidant in their solution for homogenization.

Temperature during the reaction was not quite important . The crucial point on the MDA determination may be the concentration of the homogenate. If the concentration was higher than 5%, spontaneous increase of MDA in the test tube was suspected. This result also informed that determination of MDA in the tissue always had the tendency of risky deviation on the values, probably due to autooxidation. When the brain tissue was frozen instantly and BHT was added in the homogenizing solution, the spontaneous increase was considerably prevented. So, the determination of MDA is not always the suitable parameter to detect the membrane disintegration. If other parameters of the brain edema, like water content, specific gravity, or beta-glucuronidase activity were estimated simultaneously, the determination of MDA might be of value for the etiological meaning. Therefore, MDA estimation is not considered to be the first choice parameter of the evaluating tools to describe severity of the brain edema.

The experimental model used in this study was the vasogenic type when Klatzo's definition (7) was applied. Water content in the edematous tissue increased significantly by the implantation of small amount of silver nitrate. Evans blue staining or the antibiotics penetration through blood-brain barrier was also proved previously (8). The decrease of the specific gravity in the edematous tissue explained the increase of water content. The anti-edema effect of AVS was shown on the change of the specific gravity and on the activity of beta-glucuronidase. However, the increase of MDA in the edematous tissue could not be prevented significantly by AVS administration. On this series of experiment, the determination of MDA was not sensitive enough for the therapeutic evalution against the brain edema. The estimation of lysosomal enzyme activity may be more reasonable

for the detection of the membrane dysfunction.

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