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Determination of the Level of Malondialdehyde Forming as a Result of Oxidative Stress Function in Fish

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Abstract: ***Oxidative stress resulting from degradation of the balance between free radicals in biological systems and antioxidants with a sweeping effect against them indicates an imbalance between the ability of a biological system to easily detoxify reactive intermediary products (metabolites) by producing reactive oxygen species or to repair the resulting damage. There are many protection mechanisms carried out by living beings in order to prevent oxidative damage that is caused by the effects of oxygen radicals. Fish constitute an important food chain in the ecosystem. Malondialdehyde (MDA) is a low molecular weight end product of lipid hydroperoxide degradation and is often the final product of lipid peroxidation. It is often used as a biomarker to measure the level of oxidative stress in living systems. MDA is an important reactive agent resulting from peroxidation of biological membranes. Oxidative stress in these organisms is not a disease, but it is an important factor that may cause or accelerate a disease. In this review study, the steps of MDA test that was used to measure MDA levels in fish which were exposed to nanomaterials as well as all steps including the preparation of fish tissue specimens, homogenization and centrifugation, addition of supernatant markers, incubation procedure and absorbance measurement are explained in detail..***

Keywords: Oxidative Stress, Malondialdehyde Assay, Fish, Tissue, UV Spectrophotometer

1. Introduction

Oxidative stress disturbs the oxidant-antioxidant balance of the organism. This results in the reduction of intracellular antioxidants and the intense exposure of the cells to oxygen radicals. Oxidative stress is defined as an increase in free radicals, a decrease in the level of antioxidants, or a condition which results when both occur and is harmful to the organism. Molecules, which are known as reactive oxygen species / metabolites, that form together with oxidative stress, damage cellular components especially such as lipids, proteins and DNA. in order to prevent the damage to cells caused by free radicals, all organisms in the biological system try to control the levels of free radicals [1].

Formation of oxidative stress begins with the breakdown of a hydrogen atom from methylene groups of fatty acids by a radical reactive during the oxidation of fatty acids. Polyunsaturated fatty acids (PUFA) which contain two or more double bonds are particularly susceptible to oxidation with free radicals and other highly reactive agents. In short, an allylic (bonded to the double bond adjacent carbon) hydrogen is removed by a reactive species such as the hydroxyl radical (HO •), and leads to the formation of lipid peroxyl radicals (LOO•).

This radical can then react with a second PUFA and can lead to the spread of lipid oxidation as a result of the formation of a lipid hydroperoxide (LOOH) and a second LOO•. Alternatively, LOO• may attack an intramolecular double bond and malondialdehyde (MDA) may form a degrading cyclic endoperoxide [2, 3]. Similar to other living beings, oxidative stress is not a disease for fish; but it is an important factor that can cause or accelerate the disease. Due to the absence of any significant symptom of oxidative stress, it is very difficult to detect or rehabilitate the disease in fish [4].

MDA is one of the end products of lipid hydroperoxide degradation with very low molecular weight and is often measured by the lipid peroxidation index [5]. As in other higher vertebrate organisms, lipid peroxidation or MDA in fish also results from the oxidation of unsaturated fatty acids and is the most important indicator of oxidative stress in the cellular components [6]. When studies on aquatic organisms in this context were reviewed it was revealed that in a study in which carpet embryos were exposed to the sublethal dose of pesticide Cyfluthrin (10 µg / L) for 48 days, MDA levels in brain tissues of the fish were reported to increase [7]. In an acute study in which different doses of glyphosate herbicide were applied to hybrid Amazon fish (Pseudoplatystoma sp.) for 96 hours, and their oxidative stress parameters and antioxidant defence mechanisms were evaluated. Researchers have reported that antioxidant activity is increased in liver and brain tissues of fish, and TBARS levels are elevated in liver and muscle tissues [8].

MDA is one of the low molecular weight end products of lipid hydroperoxide degradation. It is one of the final products of lipid peroxidation and is often used as a biomarker of the oxidative stress. The most common method used to assess MDA production for this purpose is thiobarbituric acid reactive substances (TBARS) marker. In this paper, the MDA protocol and all test steps that were followed in our experimental studies for the evaluation of MDA levels especially in fish exposed to nanomaterials (preparation of fish tissue specimens, homogenization and centrifugation, addition of supernatant markers, incubation process and absorbance measurement), chemicals used (TBA, BHT, buffer and acid solutions etc.) as well as all the steps involved in the application and measurement of the test are explained in detail.

2. Malondialdehyde (MDA)

The degradation of lipid hydroperoxides results in the production of bioactive aldehydes. One of the most important of these bioactive aldehydes is MDA. Because of its easy reaction with thiobarbituric acid (TBA) (Figure 1), MDA has been in use for many years as a suitable biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids [9,10]. The TBA test is based on the reactivity of the TBA with MDA to produce an intensely coloured chromogenic fluorescent red supplement product; this test was first used by food chemists to assess the autoxidative degradation of fats and oils [11]. However, the thiobarbituric acid reactive substances test (TBARS) is not noticeably a specific test, and this has raised significant questions about the use of in-vivo samples for quantification of MDA. Because MDA is one of the most widespread and reliable markers of oxidative stress in clinical situations and because of its high reactivity and toxicity, this molecule is highly popular in the biomedical research community. MDA is used to predict damage relative to reactive oxygen species [12]. MDA is one of the low molecular weight end products of lipid hydroperoxide degradation. It is one of the final products of lipid peroxidation and is often used as a biomarker of the oxidative stress. MDA is an important reactive effect due to peroxidation of biological membranes. The most common method used to assess MDA production is thiobarbituric acid reactive substances (TBARS) marker [4].

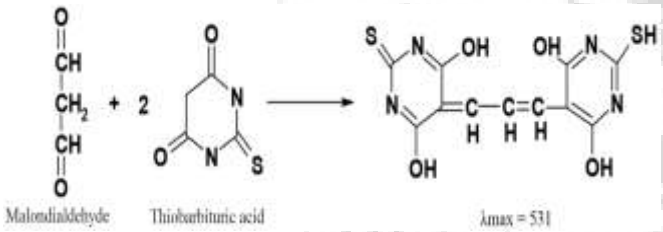

Figure 1: Reaction between malondialdehyde and thiobarbituric acid [13].

2.1 MDA Test Steps

In order to treat the samples from fish tissue organs and to reduce the oxidation of the lipids that are artificially added during the TBA reaction, butyl hydroxytoluene (BHT) and EDTA are added to the sample and reaction mixture [14, 15]. The temperature of the reaction mixture is reduced in order to minimize the dissociation of lipid hydroperoxides. Because MDA as Schiff-base compound is often bounded with protein [16], the reaction pH is optimized in order to facilitate the hydrolysis of MDA. The application of the MDA test we used as an oxidative stress marker in fish, which were exposed to nano-sized particles for these purposes, as well as all the steps leading to the measurement are described separately.

2.1.1. Preparation of fish tissue specimens

Haemoglobin which is present in the fish tissue specimens could affect the test results. For this reason, the blood in the tissue should be removed as far as possible by perfusion with the use of a suitable buffer such as phosphate buffered saline containing heparin. For this purpose, a cold test buffer or other buffer (10% w / v) of the same specification is prepared. The homogenate is removed by centrifugation and the part on the surface is stored on ice. The MDA concentration can be normalized to the wet weight of the tissue specimen or to the protein concentration of the homogenate. For this purpose, the tissue specimens, after being taken, are washed with cold water; in order to remove blood from the tissue, the specimens are exposed to sonication in 1% NaCl solution (Figure 2). If MDA analysis is not carried out immediately, samples should be shocked in liquid nitrogen and stored at -80 ° C [17].

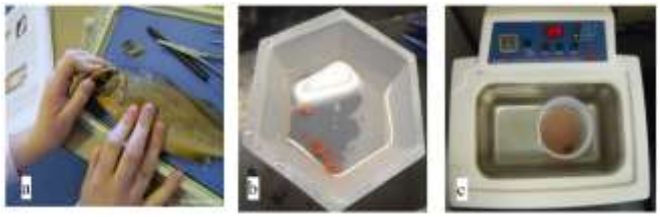

Figure 2: a) Collecting fish tissue specimens b) Washing of organs with cold water c) Removal of blood from tissues

2.1.2. Homogenization and centrifugation

The samples, which have been shocked previously, are defrosted (if stored at -80 ° C) and washed with cold water like freshly harvested organs. In order to prepare the homogenates from the tissue samples, the tissue samples are cut into smaller pieces; 100-150 mg of tissue samples are weighed out and the excess water of these pieces is removed between the two filter paper and then homogenization of the tissue specimens are handled in 2 mL of Phosphate Buffer (pH 7.2) over ice by using probed sanitary (with at least 80% power) power) (Figure 3-a, b). Homogenized samples are put in a refrigerated centrifuge working at 6000 rpm and + 4 ° C for at least 10 min. for the first precipitation process. Following the centrifugation operation, all of the liquid part remaining on the upper part of the tube (supernatant) is collected with the help of a pipette (Figure 3-c).

Figure 3: a) Homogenization of fish tissue specimens b) Shocking of tissue specimens in liquid nitrogen c) Collecting of supernatant

2.1.3. Addition of markers (reagents) in supernatant

After centrifugation process, approximately 250 µM samples from the supernatant of the specimens are transferred to a 2 mL screwed microcentrifuge tube. For the addition of the markers, 10 µL of BHT, 250 µL of acid indicator (phosphoric acid) and 250 µL of TBA are added respectively; and, at the final final stage, the tubes are completed with 2 mL of deionized water (approximately 490 µL of water is added). In addition to that, the above procedure is performed by applying markers sequentially using MDA calibrator standard 250 µL (0, 1, 2, 3 and 4 µM) in order to obtain MDA test calibration or standard Curve

graph (Figure 4).

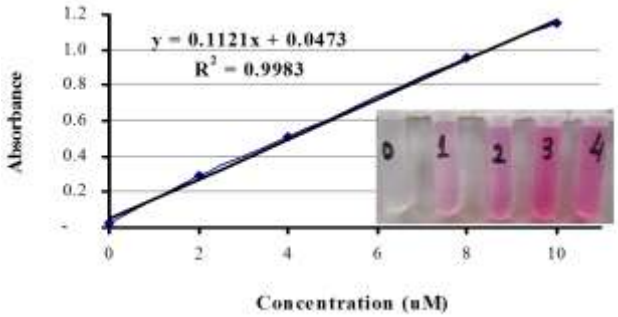

Figure 4: MDA test calibration chart

2.1.4. Incubation procedure and absorbance measurement

MDA is a secondary product of lipid peroxidation and is an important indicator used in the determination of lipid peroxidation. MDA, which can be measured by TBA, is produced as a result of the peroxidation of fatty acids containing three or more double bonds. MDA, which is the final product of fatty acid peroxidation, reacts with TBA to form a pink complex (Figure 5-a, b). Malondialdehyde forms a pink complex as a result of incubation at 70-90 ° C for 30 minutes with TBA at pH 3.4 in aerobic conditions. This complex is measured at 532 nm wavelength on a spectrophotometer [18]. For these operations; a) the samples added with reagents and MDA standards are vortexed for 1 min to ensure complete mixing b) the samples are exposed to incubation in 70-90 ° C hot water for 60 min. c) Changes in the MDA levels in tissue samples are measured spectrophotometrically according to the method modified [19]. d) After the pink colour formation in the samples is reached, the samples are cooled at room temperature, e) The micro centrifuge tubes are centrifuged for at least 30 min at 13.000 rpm (Figure 5-c). f) 1 mL sample from the supernatant part of the reaction mixture in the tubes is transferred to the UV cuvette and the absorbance values are read at 532 nm (Figure 5-d).

Figure 5: a) Incubation of the samples b) Formation of a pink coloured complex as a result of reaction between the mixture and TBA c) Centrifugation of the samples in a microcentrifuge d) Measurement of the absorbance values of the reaction mixture by UV spectrophotometer.

3. Conclusion

Lipid peroxidation is an indicator of the oxidative stress. Oxidative stress occurs as a result of the changes in the oxidant and antioxidant balance, and increases in the reactive oxygen species can be seen as oxidative damage. Free radicals, due to their high activity, can initiate lipid peroxidation by interacting with unsaturated fatty acids which are present in the cell membrane. The resulting lipid peroxides are easily reduced and can lead to the formation of many secondary products, especially MDA. The MDA test has many advantages, including being able to evaluate a large number of samples in a short period of time and economically, achieving quantitative and comparable results, and standardization of test methods. In conclusion, one of the most important features of organisms is the control of energy flow in their cells. The oxidative stress, which results from the formation of reactive oxygen species, damages biomolecules through various mechanisms. In aquatic organisms, antioxidant defence systems vary in response to environmental conditions and develop certain adaptive responses. As a bioindicator species model organism, biomarker responses such as MDA in fish tissues or organs can be used to predict the level of exposure of the ecosystem to adverse environmental conditions.

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