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# The role of Reactive Oxygen Species (ROS) in the Nandrolone-Decanoate-induced liver fibrosis

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**Abstract.** To determine the role of reactive oxygen species (ROS) and its relationship with tumor necrosis factor-alpha (TNF-alpha) during the process of developing liver fibrosis induced by Nandrolone Decanoate, 36 adult male Wistar rats are divided into 3 groups. Except for the control group, the other 2 experimental groups are injected with nandrolone decanoate (3.5 mg/kg/5 times/IM/week) for 4 weeks. After 4 weeks of injection, one experimental group (A) is decapitated. Samples from livers and plasma are obtained to conduct histopathological section, TNF-alpha ELISA, and EPR for ROS level. Then along with the same dosage of nandrolone decanoate, raxofelast (15mg/kg/day) is administrated orally to the experimental group (B) for another 4 weeks. The result of the histopathology section, ELISA, and EPR tests of group A and group B were compared. The degree of liver fibrosis, reactive oxygen species amount, and TNF-alpha level before and after the administration of antioxidants indicate the role of ROS in the development of TNF-alpha-mediated liver fibrosis. Another purpose of this experiment is to discuss the potential of antioxidants in the treatment of steroid-induced liver fibrosis.

**Keywords:** reactive oxygen species, liver fibrosis, Nandrolone Decanoate, TNF-alpha, Antioxidants

## 1. Introduction

### *1.1. Background*

Anabolic androgenic steroids (AAS) are synthetic derivatives of the male hormone testosterone. It can stimulate muscle tissue to grow in response to training by mimicking the effect of naturally produced testosterone on the body. Nandrolone decanoate, a type of AAS, is popular among athletes, especially bodybuilders, to use it at a very high dose to improve physical performance and muscle mass. The overdose would bring various harmful effects on organs. The liver is one of the organs that will be damaged by the high dosage of Nandrolone decanoate, and liver fibrosis is one of the most common hepatic injuries. Liver fibrosis is a particular circumstance with excessive accumulation of scar tissue in the liver, resulting from continuous damaged-cell repairment after subsequent liver inflammation.

TNF-alpha is an inflammatory cytokine produced by immune cells (e.g. macrophages) that can induce inflammation. It could induce sustained inflammation by triggering interleukin-1 and interleukin-6 which can introduce numerous processes that promote liver fibrosis[1]. Macrophages can also be triggered to produce free radicals to combat invading pathogens, leading to increased oxidative

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stress and inflammation [2]. A lot of research has demonstrated that the ROS and TNF-alpha have interplayed with each other in many signaling pathways[3,4]. It is now a consensual fact that TNF-alpha can increase the ROS amount due to a series of immune responses. But whether or not ROS can also downregulate the secretion of TNF-alpha remains unclear. In this study, antioxidants are used to restrict the amount of ROS after the inducement of hepatic fibrosis. TNF-alpha and fibrosis stages are monitored.

### *1.2. Previous study*

Oxidative stress can be indicated by the activities of antioxidative enzymes in tissues. Anabolic androgenic steroids have been proven can increase the oxidative stress in rats' livers indicated by enhanced lipid peroxidation extent. A significantly higher enzymatic activity was observed for AAS (stanozolol)-treated group (2mg/kg/week) on total SOD, CAT, and GPX activities than the untreated control group [5]. High oxidative stress is also found under chronic high dosage of Nandrolone Decanoate administration (10mg/kg/week) by lower antioxidant enzymatic activity in the retroperitoneal adipose tissue [6], and hepatic fibrosis is observed after 4 weeks of treatment [7]. (The opposed results in antioxidative enzymatic activity might be affected by the different substances being used, but both experiments proposed an increase in oxidative stress.)

There are several studies suggest that ROS can manipulate the production and secretion of TNF-α. However, the relationship between them is complex and context-dependent. A study published in the Journal of Immunology found that ROS produced by macrophages can inhibit the production and secretion of TNF-alpha in response to lipopolysaccharide (LPS) stimulation [8]. In the context of steroid-induced liver fibrosis, whether the ROS downregulates or has no effects on TNF-alpha remains unclear.

However, in a lot of studies, antioxidants have shown a promising future in treating steroid-induced liver fibrosis. One study published in the journal PLoS One found that treatment with the antioxidant N-acetylcysteine (NAC) was effective in reducing oxidative stress and liver fibrosis in rats treated with nandrolone-decanoate [9]. Similarly, another study published in the Journal of Steroid Biochemistry and Molecular Biology found that treatment with the antioxidant vitamin E was effective in reducing liver fibrosis in rats treated with stanozolol, another anabolic steroid [10].

### *1.3. Hypothesis*

The role of reactive oxygen species during ND-induced hepatic fibrosis remains undetermined. In respect of the fact that hepatic fibrosis can be reversed after the withdrawal of nandrolone decanoate in 4 weeks, the administration of antioxidants could eliminate or at least relieve the condition of fibrosis due to its effect on reducing oxidative stress. If the liver fibrosis stage has regressed, it indicates that ROS plays an essential role in reinforcing the development of fibrous tissue. The TNF-alpha level before and after the treatment of raxofelast (antioxidant) can indicate the relationship between TNF-alpha and ROS.

## 2. Experiment methods

### *2.1. Animals*

36 male adult Wistar rats are cultured under the same air humidity, room temperature, and controlled light. They are randomly divided into 3 groups (n=12). Except for the control group, Nandrolone Decanoate is injected into the thigh muscles of the hind limb with a dosage of 3.0 mg per kg bodyweight. 5 injections are conducted each week. After 4 weeks of injection, the control and one experimental group (A) are killed by decapitation. The liver is excised and maintained immediately in liquid nitrogen at −80°C if the following tests can not be performed immediately, and the blood samples are collected [11]. Maintain the injection for the remaining experimental group (B) of the same dosage of ND, along with the oral administration of raxofelast in a dosage of 15mg per kg bodyweight daily.

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### *2.2. ROS Qualification-Electron Paramagnetic Resonance (EPR)*

Electron Paramagnetic Resonance is a direct method to detect free radicals in vivo and also in vitro conditions. Reactive oxygen species are easily degraded into particles that contain free unbound electrons which can be effectively detected by EPR. EPR testing is conducted as soon as the liver tissue is excised. Calibration samples have been prepared from blood and liver as described in Dumitrescu, Sergiu D [12]. The level of oxidative stress is monitored by EPR on liver tissues.

### *2.3. Histopathological section*

The histopathological section provides a direct evaluation of the stage of liver fibrosis. Details of the histopathological approach to rat liver tissue are described by Shakti Pattanayak [13]. As described in the protocol, permanent slides are preserved for further comparison and analysis.

### *2.4. Enzyme-Linked Immunosorbent Assay*

TNF-alpha as an important pro-inflammatory mediator will circulate in the bloodstream induced by macrophages. It will be detected by the receptors in the target tissue. In this experiment, an ELISA kit is used to detect the TNF-alpha level in the serum sample of rats. Specific procedures are described in Petrovas, Constantinos [14].

## 3. Result

### *3.1. EPR*

In the EPR spectrum after 4 weeks of injection, the experimental group A shows several peaks of different free radical molecules compared to the control group. This indicates the elevated level of reactive oxygen species induced by ND injection. After another 4 weeks of administration of antioxidants along the ND, the oxidative stress level should have reduced compared to the data from group A.

### *3.2. Histopathological Section*

The histopathological sections from group A rats' livers show collagen deposition [15]. The fibrous expansion of the group A samples occupies the most portal areas. Some portal-to-portal bridging as well as portal-to-central bridging are observed. If the hypothesis is validated, in the histopathological sections in group B liver samples a reduction in collagen deposition and fibrosis measurement should be observed, which can be indicated by the less fibrosis-occupied portal areas. If the hypothesis is invalidated, there should be no relief of the fibrosis symptom observed. The detailed stages of liver fibrosis development are shown in Figure 1.

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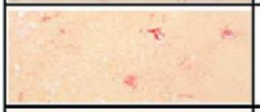
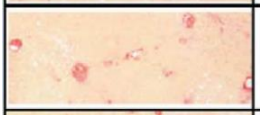
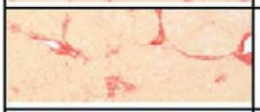
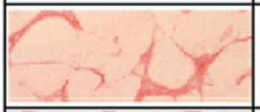
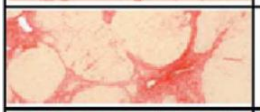
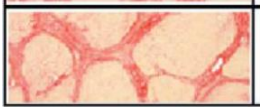
| Appearance | Ishak stage: Categorical description | Ishak stage: Categorical assignment | Fibrosis measurement* |
|---|---|---|---|
|  | No fibrosis (normal) | 0 | 1.9% |
|  | Fibrous expansion of some portal areas ± short fibrous septa | 1 | 3.0% |
|  | Fibrous expansion of most portal areas ± short fibrous septa | 2 | 3.6% |
|  | Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging | 3 | 6.5% |
|  | Fibrous expansion of portal areas with marked bridging (portal to portal (P-P) as well as portal to central (P-C)) | 4 | 13.7% |
|  | Marked bridging (P-P and/or P-C), with occasional nodules (incomplete cirrhosis) | 5 | 24.3% |
|  | Cirrhosis, probable or definite | 6 | 27.8% |

Note: *Proportion (%) of the area of the illustrated section showing Sirius red staining for collagen (collagen proportionate area).

**Figure 1.** Stage component of the Ishak system [16].

### *3.3. ELISA*

The results of the ELISA kit on the serum sample of group A rats show an elevated TNF-alpha level compared to the control group. After 4 weeks of administration of raxofelast, the TNF-alpha level could be either increased, decreased, or unchanged. The amount of TNF-alpha would be represented by the intensity of a detectable signal (usually colorimetric) that is proportional to the amount of the target molecule in the sample.

## 4. Future Direction

In this experiment male mice are used as the experiment subject, which is reasonable due to male have more stable hormone condition which might limit the confounding variables. However in the actual circumstances, steroid usage is increasing among female population. One possible approach in the future is select female mice as the experiment subject and see if the results vary.

The way to assessing the liver fibrosis in this experiment is histopathological section, which is currently considered the gold standard for assessing liver fibrosis. However, interpretation of histopathological sections can be subjective, as it depends on the experience and skill of the observer. Different observers may interpret the same section differently, which can lead to some degree of variability in the results. In the future studies, one might consider assessing the fibrous stage aided by other measurement like blood tests.

ELISA is a widely used and standardized method for measuring TNF-alpha levels in liver tissue homogenates. However it may be subject to interference from other cytokines or matrix components present in the sample. Immunohistochemistry is a method that requires staining the liver tissues and

enables researchers to visualize the TNF-alpha in tissues. While it might provide more accurate observation of TNF-alpha level, it requires skilled interpretation by a pathologist.

## 5. Conclusion

Both TNF-alpha and ROS are proven to be the essential factors in the process of steroid-induced liver fibrosis. Whether or not the antioxidant administration works will indicate the role of ROS in this process. Does ROS play a determining role during the development of hepatic fibrosis? It also implies the relationship between ROS and TNF-alpha. Since in this experiment, the application of antioxidants, raxofelast, is the only variables between two experimental groups while all other conditions are under controls. How TNF-alpha level changes after 4 weeks additional injection of antioxidants can indicate how these two substances interact during the process. If the fibrous expansion is limited because of the administration of antioxidants, the raxofelast synthetic can be a promising health product for athletes and bodybuilders who are under massive dosages of AAS. In respect of the fact that some people might not be willing to seek physicians or pharmacists for their AAS intake circumstance, antioxidants can be considered as assisted and accessible health products to be taken along with in order to limit the drawbacks of overdosing AAS in the liver and maybe other susceptible tissues.

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