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Submandibular Salivary Gland Vulnerability: Unveiling Aflatoxin B1-Induced Toxicity and Rosmarinus Officinalis 's Protective Role

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KEYWORDS

Abstract

Aflatoxins (AF) represent an extremely toxic and carcinogenic mycotoxin family produced by specific molds, notably Aspergillus flavus and Aspergillus parasiticus[1, 2]. These molds are prone to infiltrate various food crops, particularly grains, nuts, and legumes, both prior to and following harvest under suitable growth conditions. Among the various types of aflatoxins, Aflatoxin B1 (AFB1) holds prominence as one of the extensively studied carcinogens, inflicting detrimental health consequences on both humans and animals. It stands as one of the most potent naturally occurring carcinogens. The notoriety of AFB1 extends to its capability to induce severe and harmful toxic effects across various organs. This cumulative evidence led the International Agency for Research on Cancer to classify AFB1 as a Group I carcinogen[3].

The mechanism of action of aflatoxin B1 (AFB1) involves its metabolism and interaction with cellular components, particularly DNA resulting in DNA damage and mutations. AFB1 can affect cellular signaling pathways, potentially leading to uncontrolled cell growth and proliferation. It can interfere with the function of tumor suppressor genes and oncogenes, further contributing to carcinogenesis[4]. Furthermore, many studies showed that AFB1 induces oxidative stress through formation of reactive oxygen species (ROS) which exceeds the antioxidant activity of the natural enzymatic and non-enzymatic components of the cells. Therefore, this increased oxidative stress causes deleterious effect on the cell organelles particularly the nuclei and mitochondria and can damage cellular components such as DNA, proteins, and lipids. ROS contribute to oxidative stress, inflammation, and cellular dysfunction[5].

Introduction

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Journal of Chemical Health Risks

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Furthermore, numerous investigations have demonstrated that AFB1 functions as both an initiator and promoter of the apoptotic pathway. Apoptosis, a programmed mechanism of cell demise, is crucial for maintaining cellular and tissue equilibrium and is implicated in the development of various diseases. Additionally, extensive research substantiates that aflatoxins interact antagonistically with diverse cellular proteins, resulting in inhibition of carbohydrate and lipid metabolism, as well as protein synthesis. These interactions have the potential to initiate apoptosis, as indicated by various studies[6, 7].

Certain proteins regulate the apoptotic process and are either pro or anti apoptotic proteins. Caspase-3 and Bax are types of pro-apoptotic proteins, while Bcl-2 is considered an anti-apoptotic regulator. Caspase-3 is an enzyme that plays a central role in the execution phase of apoptosis. It plays a pivotal role in executing the actual breakdown of cellular components, encompassing DNA, cytoskeletal proteins, and a variety of other substances. This process culminates in the systematic disassembly of the cell into apoptotic bodies, which phagocytic cells then efficiently remove, avoiding inflammation or harm to nearby tissues [8,9]. Maintaining the balance of caspase-3 activity is vital for sustaining tissue balance, facilitating embryonic growth, and securing the immune system's effective operation. An imbalance in caspase-3, along with other caspases, can result in a range of illnesses, such as cancer, neurodegenerative diseases, autoimmune disorders, and developmental irregularities. Due to its pivotal role in apoptosis, caspase-3 has emerged as a key subject in scientific studies focusing on deciphering cell death processes and investigating possible treatment strategies [10]. Caspase-3 and Bax are important elements in the apoptotic process and can be activated by either an extrinsic or intrinsic. The intrinsic pathway is regulated by the mitochondria, therefore AFB1 which markedly causes mitochondrial damage, might be responsible for the increased cellular apoptosis[9, 11].

In recent years, there has been a growing focus on natural compounds as potential agents to mitigate the deleterious impacts of AFB1. The scientific community's attention has notably shifted toward exploring the shielding capabilities of antioxidants against the adverse effects of aflatoxins[12-14]. Notably, within the realm of medicinal plants, phenolics have garnered significant interest as primary natural antioxidants. These phenolics comprise a diverse group of approximately 8000 compounds, including flavonoids. Among the botanical species recognized for their substantial flavonoid content is Rosmarinus Officinalis (RO). Multiple studies have demonstrated the affirmative biological effects of RO, particularly regarding its antioxidant activity, which serves to ameliorate the detrimental consequences associated with AFB1 exposure[15, 16].

Salivary glands are essential components of the human oral cavity, contributing to digestion, hydration, and the maintenance of oral health. The submandibular salivary glands, in particular, play a crucial role in producing saliva rich in enzymes and mucins that aid in the initial digestion of carbohydrates and protect oral tissues[17, 18]. However, these glands are not immune to the adverse effects of systemic diseases[19], drugs[20], and environmental toxins[21, 22]. Recent research has begun to unveil the intricate interplay between AFB1 exposure and the structural, functional, and biochemical integrity of these glands[13, 23].

Considering that the saliva generated by the salivary glands serves a crucial role in upholding oral health and physiological balance, and owing to its potent antioxidative attributes that establish the foremost line of antioxidant defense within the gastrointestinal tract against reactive oxygen species (ROS), the objective of this research was to explore the potential of Rosmarinus Officinalis to counteract the adverse impacts of AFB1 on the submandibular salivary glands in adult male Albino rats.

Materials and methods

A cohort of twenty-one healthy adult male Albino rats, aged three months and weighing between 200-220 grams, constituted the study subjects. Sample size determination utilized G*Power version 3.1.9.2 (University Kiel, Germany). With an effect size of 0.95, α significance level of 0.05, and β level of 0.05 (achieving a power of 95%), the calculated sample size (n) amounted to 21 samples distributed across three groups. The experimental design employed in this study received ethical approval from the Research Ethics Committee at The British University in Egypt and experimental procedures were performed in the Centre of Innovative Dental Sciences.

The rats were individually placed in separate cages, with each cage accommodating five rats. These cages were situated in a controlled environment featuring a

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consistent temperature of 25°C, humidity levels spanning from 45% to 75%, and adhering to a standard photoperiod of 12 hours of light succeeded by 12 hours of darkness. Throughout the entire experimental duration, the rats were provided with a natural diet and unrestricted access to drinking water. The allocation of rats to different groups was performed randomly. The specific groups were as follows: Group I, referred to as the control group, received intraperitoneal injections of 9% saline on a daily basis for a duration of 4 weeks. Group II, known as the AFB1 group, underwent intraperitoneal injections of AFB1 (at a dose of 2 mg/kg) once daily for a span of 4 weeks[13]. Group III, designated as the AFB1+RO group, experienced intraperitoneal injections of AFB1, maintaining the same dosage and duration as Group I, followed by intraperitoneal injections of RO extract (at a dosage of 400mg/kg) for a subsequent period of 2 weeks[13].

RO methanolic extract

The dried leaves of Rosmarinus Officinalis (RO) were sourced from Harraz for Food Industry, Natural Products, and Botanical Herbs (Product # 215). These dried leaves underwent grinding to facilitate the subsequent methanolic extraction, following the methodology outlined in previous studies[24].

Upon completion of the experimental timeline, the animals within each experimental group were humanely euthanized through cervical dislocation. The submandibular salivary glands from both the right and left sides of each animal were carefully dissected. These extracted submandibular salivary glands underwent the necessary preparation for electron microscope analysis. Additionally, an immuno-peroxidase staining technique was employed to facilitate the immunohistochemical localization of Caspase-3 within the glandular tissue. This staining procedure involved the use of Anti-Caspase-3 antibody, and the incubation of the tissue sections with the staining reagents.Negative controls were methodically established. In these controls, the primary antibody was substituted with a nonspecific serum possessing the same dilution as the respective antibody.

Results

Ultrastructural Results:

Group I (Control group):

Analysis using transmission electron microscopy of the rat submandibular salivary glands in the control group revealed detailed structural and ultrastructural characteristics (Figure 1). A highly developed network of rough endoplasmic reticulum (RER) formed around the nuclei. The acinar cells contained abundant apical electronlucent secretory granules of varying sizes, containing finely granular electron-dense flocculent substances. The intercellular spaces were filled with membranous folds exhibiting lateral interdigitation. The nuclei of the acinar cells appeared round with differing electron densities.

The RER manifested as an interconnected membranous system, extending its cisternae uniformly throughout the cytoplasm in parallel formations. Golgi bodies, recognizable by their stacked flattened pouches, were situated within the cytoplasm adjacent to the endoplasmic reticulum and in proximity to the secretory granules. Oval-shaped mitochondria were evenly dispersed throughout the entire cytoplasm.

The striated ducts displayed distinct characteristics, including profound infoldings of the basal plasma membrane and numerous longitudinally oriented mitochondria recognizable by their cross-sectional cristae. The centrally rounded nuclei exhibited evenly distributed granular chromatin. In the proximal segment of the striated ducts, known as the granular convoluted tubule segments, tall columnar cells lined the interior, densely packed with membrane-bound secretory granules of varying sizes and densities. The nuclei, also rounded, displayed diverse electron densities, with the peripheral region being more concentrated in heterochromatin. Numerous mitochondria of assorted sizes, identifiable by their discernible cristae, were dispersed extensively throughout the cytoplasm.

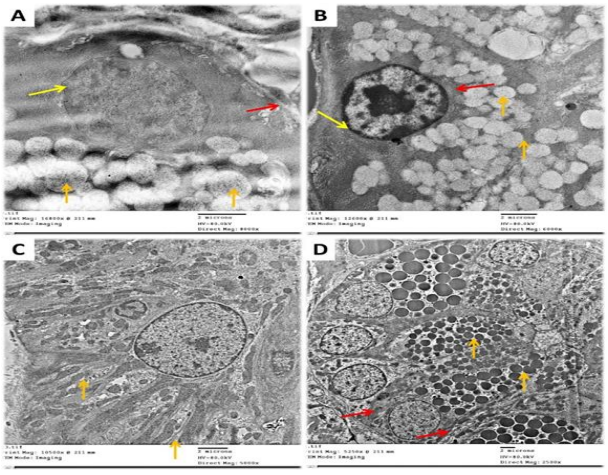

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Figure 1. Electron photomicrographs of submandibular salivary gland of rat of group I (control group) (a) showing well developed RER (yellow arrow), secretory granules (brown arrows) and intercellular spaces (red arrow) (Uranyl acetate & lead citrate X8000), (b) showing rounded nucleus (red arrow), well developed RER (yellow arrow) and secretory granules (brown arrows) (Uranyl acetate & lead citrate X8000), (c) showing striated duct cell with longitudinal arranged mitochondria (brown arrows) and intercellular spaces (red arrow) (Uranyl acetate & lead citrate X5000), (d) showing granular convoluted tubule with high number of membrane bound secretory granules (brown arrows) and mitochondria (red arrow) (Uranyl acetate & lead citrate X2500).

Group II (AFB1 group):

Examination using electron microscopy of the rat submandibular salivary glands subjected to AFB1 exposure unveiled discernible changes in the ultrastructure of both the secretory terminal sections and the duct system when juxtaposed with the control group (Figure 2). Furthermore, the presence of shrunken apoptotic nuclei was observed in secretory cells, characterized by nuclear indentation and lobulation, accompanied by chromatin margination and aggregation. The mitochondria exhibited an expanded appearance with disintegrated cristae, resulting in nuclear indentation. The rough endoplasmic reticulum (RER) demonstrated significant dilation, and the cisternae of the RER retained secretions linked with compromised mitochondria. These dilated RER cisternae contributed to a vesicular appearance within the cytoplasm. Additionally, the arrangement of secretory granules appeared less compacted. In this group, the cells of the striated duct displayed conspicuous reductions in both the height and density of radially arranged mitochondria. Furthermore, noticeable cytoplasmic vacuolization areas emerged, concomitant with a decrease in the quantity of apical secretory granules. An initiation of nuclear chromatin clumping was also evident in the nuclei. The granular convoluted tubule segments exhibited a marked reduction in both the size and number of apical secretory granules, with extensive cytoplasmic vacuolization areas being prominently displayed.

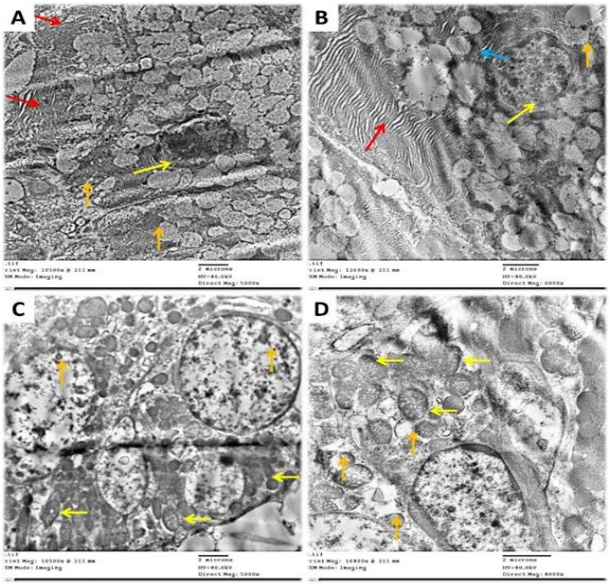

Figure 2. Electron photomicrographs of submandibular salivary gland of rat of group II (AFB1 group) (a) showing shrunken nuclei (yellow arrow) swollen mitochondria (brown arrow) and dilated cisternae of RER (red arrows) with retained secretion (blue arrow) (Uranyl acetate & lead citrate X5000), (b) showing shrunken nuclei (yellow arrow) swollen mitochondria (brown arrow) and dilated cisternae of RER (red arrows)(Uranyl acetate & lead citrate X6000), (c) showing striated duct cell with reduced size of radially arranged mitochondria (yellow arrow) nuclear chromatin clumping of the nucleus (brown arrow) (Uranyl acetate & lead citrate X6000), (d) showing granular convoluted tubules with marked reduction in the size and number of the apical secretory granules (yellow arrow) Wide areas of vacuolization in their cytoplasm (brown arrow) (Uranyl acetate & lead citrate X6000).

Group III (AFB1 + RO)

Group III:

In comparison to the ultrastructural observations made in the AFB1-exposed group, the submandibular salivary glands of rats in the group immediately treated with a 2-week regimen of Rosmarinus Officinalis extract (at a dosage of 400mg/kg) exhibited a marked enhancement in the structural characteristics of the glands (Figure 3). The secretory acini, enveloped by myoepithelial cells, appeared to have regained their customary architecture. The configuration of rough endoplasmic reticulum (RER), forming parallel sacs encircling the nucleus,

Journal of Chemical Health Risks
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JCHR (2023) 13(6), 2315-2322 | ISSN:2251-6727

displayed only subtle indications of cisternal expansion. The oval-shaped mitochondria retained their typical dimensions, while the semicircular stacked flat sacs of the Golgi apparatus positioned themselves between the pattern of RER and the secretory granules. The nucleus showcased an orderly dispersion of chromatin, with the chromatin being distributed evenly throughout. This arrangement was complemented by a centrally located, circular nucleolus. Extensive vacuolization was evident, although it manifested to a lesser degree within the cytoplasm.

In the context of the striated ducts, the infoldings of the basal membrane exhibited partial restoration, creating separations for radially oriented mitochondria. These mitochondria, although not achieving the size and number characteristic of the control group, appeared more abundant compared to the AFB1-exposed group. Similarly, the granular convoluted tubules demonstrated an elevated presence of apical secretory granules, forming a cluster of electron-dense granules of varying sizes occupying the cell's apical pole. The nuclei displayed a rounded shape with normal chromatin distribution.

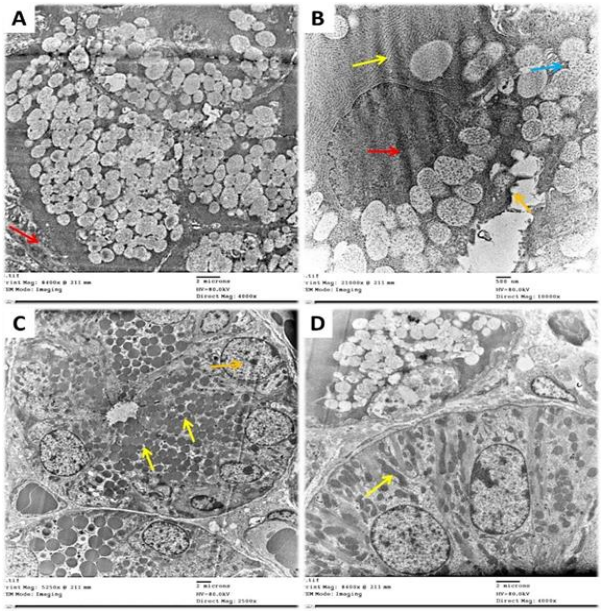

Figure 3. Electron photomicrograph of submandibular salivary gland of rat of group III (AFB1 + RO) (a) showing the architecture of secretory cells and associated myoepithelial cell (red arrow) (Uranyl acetate & lead citrate X4000), (b) showing the secretory cell with normal array of RER (yellow arrow), oval bodies of mitochondria (brown arrows), the semicircular stacked flat sacs of Golgi apparatus (blue arrow), the regular arrangement of dispersed nuclear chromatin and a central round nucleolus (red arrow) (Uranyl acetate & lead citrate X10000), (c) showing granular convoluted tubules with increased amount of secretory granules (yellow arrow) and normal chromatin distribution of the nucleus (brown arrows) (Uranyl acetate & lead citrate X2500), (d) showing striated duct with more well oriented mitochondria (yellow arrow) (Uranyl acetate & lead citrate X4000).

3-Caspase 3 immunoreactivity:

The histological sections of the AFB1 + Rosmarinus Officinalis (RO) treated group exhibited a notable reduction in Caspase 3 antigen expression within acinar cells, presenting a clear contrast with the AFB1 group. Conversely, a mild decrease in immunoreactivity towards the proliferation antigen was evident in the ductal profiles. However, this immunoreactivity appeared to closely resemble that of the control group (Figure 4).

Results of the Post Hoc Tukey HSD test, comparing the various groups, underscored the statistical significance of differences. Notably, a substantial increase in the mean area percentage of Caspase 3 immuno-expression was observed in the AFB1 group compared to the control group ($p < 0.0001$). Similarly, a noteworthy difference ($p < 0.0001$) was discerned between the AFB1 and AFB1 + RO groups. Conversely, no significant difference ($p = 0.0173$) was observed between the control group and the AFB1 + RO group (Figure 5).

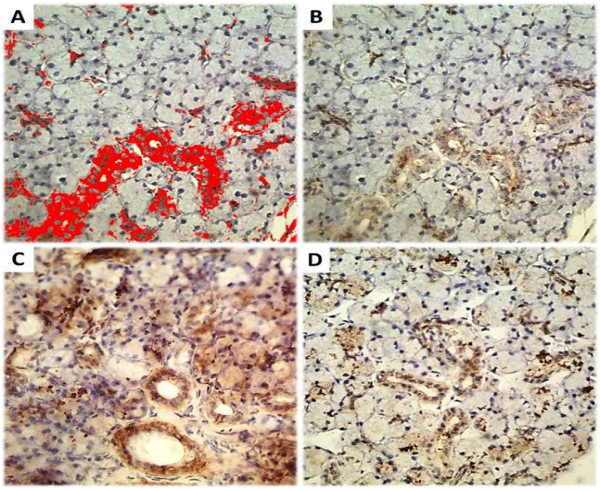

Figure 4. Photomicrographs of submandibular salivary gland showing (a) Example for Caspase-3 immune

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JCHR (2023) 13(6), 2315-2322 | ISSN:2251-6727

reaction measeurement using Image J software, (b) mild Caspase-3 immune reaction in the acinar and ductal cells of control group, (c) Severe Caspase-3 immune reaction in the acinar and ductal cells of AFB1 group, (d) mild Caspase-3 immune reaction in the acinar and ductal cells of AFB1+RO group (X400).

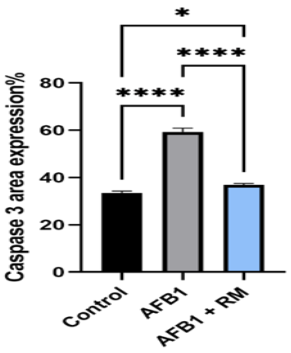

Figure 5. Bar chart representing mean and standard deviation of Caspase-3 area expression. * indicates significance ($p < 0.05$), **** indicates significance ($p < 0.0001$) for each group compared to the control group and differences between groups.

Discussion:

While the primary concern regarding aflatoxin B1 (AFB1) has historically revolved around its hepatotoxic and carcinogenic effects[25], recent studies have begun to explore its impact on non-hepatic organs[26, 27]. The submandibular salivary glands, crucial components of the oral cavity, have been gaining attention due to their role in maintaining oral health and their susceptibility to various environmental stressors. The link between AFB1 exposure and submandibular salivary gland toxicity introduces a new dimension to the understanding of mycotoxin-related health risks.

In the present study, histopathological investigations have provided valuable insights into the morphological changes induced by AFB1 in submandibular salivary glands. Cellular degeneration, inflammatory infiltrates, and fibrotic changes have been observed, suggesting that AFB1 disrupts the normal structure and function of the glands. These findings align with the growing evidence of the mycotoxin's systemic effects beyond the liver, highlighting the importance of considering extrahepatic target organs when assessing AFB1 toxicity.

Many studies showed that AFB1 induces oxidative stress through formation of ROS which exceeds the antioxidant activity of the natural enzymatic and non-enzymatic components of the cells[28, 29]. Some researchers stated that the intake of AFB1 in contaminated food may lead to inhibition of DNA/RNA synthesis process which in turn will directly cause protein synthesis inhibition. They cause separation between the ribosomes and the endoplasmic reticulum through affection of endoplasmic reticulum membrane binding site for ribosomes[30].

Our own findings align with previous research, highlighting the detrimental effects of AFB1 on salivary glands[13, 23]. Transmission electron microscopy (TEM) analysis exposed cytoplasmic vacuolization and mitochondrial disintegration. Additionally, early signs of cell injury were evident, including nuclear chromatin clumping and diminished mitochondrial density. These findings are in harmony with the initiation of apoptosis—a programmed cell death mechanism—within AFB1-exposed submandibular salivary glands, indicated by Caspase-3 activation.

Exposure to certain toxins, such as AFB1, can trigger apoptotic pathways. AFB1-induced DNA damage and oxidative stress can trigger signaling pathways that initiate apoptosis as a defensive mechanism to destroy cells that have the potential to develop cancer as a result of DNA alterations. Caspase-3, functioning as an executor caspase, is frequently triggered inside these apoptotic pathways to perform the disassembly of cellular constituents [31].

The current study reveals that a rise in caspase-3 levels indicates the activation of apoptosis and the ongoing process of programmed cell death. This response aims to eliminate cells that have sustained substantial damage, thereby reducing the chances of these damaged cells evolving into cancerous forms or causing other health issues. Therefore, the observed increase in caspase-3 levels after exposure to AFB1 represents a biological defense mechanism, where the cell attempts to mitigate possible harm by removing affected cells through the process of apoptosis.

Our study found that the administration of Rosmarinus Officinalis (RO) resulted in a notable improvement in glandular architecture, as assessed by transmission electron microscopy (TEM). This observation aligns with the findings of other research that have shown the exceptional antioxidant capabilities of RO [32-34]. The power of RO to neutralize reactive oxygen species, functioning as a strong scavenger of free radicals, establishes it as a promising candidate for therapeutic use. Furthermore, the decrease in the expression of

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Caspase-3 antigen in acinar cells, in comparison to the group exposed to AFB1, indicates a decrease in proapoptotic activity, which is a positive indication of the protective impact of RO.

The robust antioxidant properties of RO are largely due to its abundant content of isoprenoid quinones. These compounds act as effective terminators of free radical chains and also serve as efficient chelators, neutralizing reactive oxygen species (ROS) [32]. Additionally, key constituents of RO, particularly carnosic acid and carnosol, are identified as highly effective in scavenging peroxyl radicals. The distinctive combination of isoprenoid quinones with carnosic acid and carnosol underscores RO's significant capability to counteract harmful radicals and highlights its potential in effectively reducing damage from oxidative stress [35].

In summary, the impact of AFB1 extends beyond liver-related complications, affecting the submandibular salivary glands as well. The toxic effects of AFB1 compromise the structural and functional integrity of these glands, as evidenced by histological and ultrastructural examinations. Rosmarinus Officinalis shows promise as a protective measure due to its antioxidant properties, which have been observed to promote structural restoration and regulate apoptosis in the glands. Further research is necessary to fully comprehend the intricate mechanisms behind RO's efficacy. This could pave the way for novel treatments aimed at mitigating AFB1-induced harm and preserving the health of the submandibular salivary glands.

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