Examination of Apoptotic and Autophagic Effects of Chronic Roumilast Use on Rat Testicular Tissue by Immunohistochemical and Immunofluorescence Methods

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Abstract

Roumilast (ROF) (3-cyclo-propylmethoxy-4-difuorome-thoxy-N-[3,5-di-chloropyrid-4-yl] benzamide) is a second generation and forcible phosphodiesterase-4 (PDE4) inhibitor. This study aims to investigate the effects of chronic Roumilast in different doses on testicular tissue and testosterone levels in healthy Sprague-Dawley rats.

During the research, the 6 weeks old (180–200 gr), 36 male rats were divided into 4 groups. Roumilast (ROF) was administered as 0.5 mg/kg and 1 mg/kg by oral gavage for four weeks, once each day. Hematoxylin-Eosin (H&E) staining for histopathological examinations in testicular tissue, immunohistochemical and immunofluorescence examinations for Caspase-3, Apoptosis Inducing Factor (AIF) and Light Chain 3β (LC3B) expression levels, and ELISA method used to determine serum testosterone levels. Data were analyzed using SPSS v.22 with Kruskal-Wallis, Mann Whitney-U, and Wilcoxon tests.

Roumilast group lost weight compared to the control and sham. Shedding in the seminiferous epithelium, degenerations in the interstitial area, separation between cells, desquamation, interstitial edema and degenerative changes in the testicular tissue was observed. While apoptosis and autophagy determined by Caspase-3, AIF and LC3B were close and statistically insignificant in control and sham, there was significantly increased apoptotic and autophagic changes and immunopositivity in the ROF groups. The 1 mg/kg Roumilast group’s serum testosterone level was lower than control, sham and 0.5 mg/kg Roumilast groups.

When the research data was evaluated, it was determined that the chronic use of the active ingredient Roumilast, which has a broad-spectrum area such as COPD, arthritis, neurodegenerative diseases, liver and dermatology, had negative effects on the testicular tissue and testosterone level of rats.

Introduction

Roumilast (ROF) (3-cyclo-propylmethoxy-4-difuorome-thoxy-N-[3,5-di-chloropyrid-4-yl] benzamide) is a second generation and forcible phosphodiesterase-4 (PDE4) inhibitor (Hatzelmann et al. 2010). Phosphodiesterases (PDEs) are phosphohydrolase enzymes that cause hydrolysis of the 3'-phosphate bridges of intracellular secondary messenger molecules, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), that is, they regulate the continuity and termination of cAMP and cGMP signals in the cell (Drobnis and Nangia 2017). As PDEs have a critical effect on signaling pathways in various pharmacological processes, including cell function, they have drawn the attention of researchers relevant to many disease pathologies (Ouyang et al. 2021). PDEs, which are a large family of enzymes, reportedly consist of 11 subtypes (PDE1-PDE11) and over 40 isoforms (Nabavi et al. 2019).

Phosphodiesterase inhibitors such as roflumilast act by inhibiting the breakdown of intracellular cAMP, reducing inflammation, suppressing cell proliferation, cytokine production, and chemotaxis. Roflumilast, which was used as an antidepressant in the 1980s, is the first selector PDE4 inhibitor approved for
human usage in 2010, with the primary indication being chronic obstructive pulmonary disease (COPD) (Giembycz and Field 2010; Wedzicha 2013). In the later years, it started being used in the fields of arthritis, neurodegenerative diseases, liver and dermatology (Fala 2015; Nabavi et al. 2019; Fleming et al. 2020).

Apoptosis and autophagy are phenomena that occur in many cases as cell death mechanisms (Zheng et al. 2016). Apoptosis is the mechanism in which the cell self-destructs, regulated by genes, needs protein synthesis and energy, and maintains the balance in the organism (Voss and Strasser 2020). This mechanism of death is divided into two as caspase-dependent and caspase-independent apoptosis. In the caspase-dependent apoptosis, caspase-3 is in caspase-independent apoptosis; To induce programmed cell death, the AIF protein plays a role in the initiation of the caspase-independent apoptotic pathway by triggering chromatin condensation and DNA fragmentation in the cell and regulating the permeability of the mitochondrial membrane (Yu et al. 2015). Autophagy, on the other hand, is a catabolic mechanism that sends not only cytosolic proteins, but also intracellular cytosolic components, organelles and aggregates to lysosomes for degradation, prevents the accumulation of misfolded proteins and destroys unwanted organelles (García-Prat et al. 2016). LC3B protein, which is a reagent of autophagy, is effective in initiating the autophagic pathway (Herb, Gluschko, and Schramm 2020). Studies have shown that apoptosis and autophagy interact with Roumilast (Bajpai et al. 2020).

Usage doses of Roumilast have been determined as 250, 500 and 1000 µg/kg, and it is reportedly more effective in medium and high doses (Murad et al. 2017). In the pharmacology review published by the FDA in 2011, it was reported that headache, gastrointestinal disorders, dizziness, palpitations, flu, and arterial hypotension were observed after a single oral application of 2.5 and 5 mg in Phase 1 studies of Roflumilast (FDA 2011).

When the studies are examined in the literature, the protective properties of Roflumilast in different tissues have been reported against experimentally induced damages and some toxic agents. However, there is no in-vivo scientific study to investigate healthy testicular tissue toxicity after chronic use. Chronic use of Roflumilast, which has a widespread clinical use, was found to be apoptotic, caspase-dependent (Caspase-3), caspase-independent (AIF), autophagic, expressions of (LC3B) proteins and their effects were investigated by analyzing serum testosterone levels on rat testicular tissue with this study.

**Materials And Methods**

In the study, a total of 36 male 40 days old, 180–200 g, Sprague-Dawley rats were used. The groups were randomly formed. They were fed as *ad libitum* with tap water in an environment with 12 hours of darkness and 12 hours of lighting and kept in standard cages. The ROF groups were given 0.5 and 1 mg/kg Roflumilast by oral gavage at the same time every day for 4 weeks (Table 1) (Botros et al. 2020).
### Table 1
Experiment Groups.

| Groups               | N  | Application                                |
|----------------------|----|--------------------------------------------|
| Control              | 8  | None                                       |
| Sham                 | 8  | 1 ml physiological saline solution         |
| 0.5 mg/kg ROF group  | 10 | 0.5 mg/kg Roumilast                         |
| 1 mg/kg ROF group    | 10 | 1 mg/kg Roumilast                          |

At the end of the experiment, rats were deeply sedated with Sevoflurane (Sevorane®, Abbott Lab. Istanbul, Turkey) and cervical dislocation was performed, and intracardiac blood and testicular tissue samples were collected. Body weights and testes weights of rats in all groups were measured. Testicular tissue samples were placed in 10% buffered neutral formalin solution, and after routine histological procedures, they were blocked in paraffin.

**Histological Studies**

H&E staining technique was applied to the sections taken from the blocks to examine the general structure of testicular tissue. The preparations were examined under the light microscope (Nikon BX51), 6 selected fields chosen randomly, and the experimental groups were compared by scoring according to the Johnsen criteria in order to reveal the possible severity of Roumilast damage to the testicular seminiferous tubule cells (Johnsen 1970; Erboga et al. 2016).

**Immunohistochemical Studies**

The 5 µm sections taken from the blocks were passed through xylol and alcohol series and washed with PBS for 10 minutes in 3% H2O2 and treated with antigen retrieval solution. Afterwards, a protein block was applied to completely cover the sections. Then the sections of Table 2 from were incubated with the indicated primary antibodies. After applying Mouse and Rabbit Specific HRP/DAB IHC Detection Kit-Micro-polymer kit (Abcam, Catalog No. ab236466) as secondary antibody and 3–3’Diaminobenzidine (DAB) as chromogen to tissue samples, counterstaining was performed with Mayer’s Hematoxylin. Tissue samples were passed through graded alcohols and xylol series and sealed with entellan.
Table 2
Primary antibody information used in immunohistochemical analyses.

| Primary Antibody      | Company and Catalog No.                      | Dilution rate | Duration and temperature          |
|-----------------------|----------------------------------------------|---------------|-----------------------------------|
| Cleaved – Caspase-3†  | Cell Signaling Company, Catalog No. 9661     | 1/200         | 20 m at room temperature          |
| AIF – 1 polyclonal‡   | Aviva Systems Biology, Catalog No. OAGA04280 | 1/200         | 20 m at room temperature          |
| LC3B polyclonal‡      | Abclonal, Catalog No. A7198                  | 1/200         | 20 m at room temperature          |

† Used to determine caspase-dependent apoptosis
‡ Caspase was used to determine independent apoptosis
§ Used to determine autophagy

Immunofluorescence Staining Method

The procedures specified in immunohistochemical staining were applied to the prepared sections until incubation with primary antibody. The sections were then incubated at 37°C for 45 minutes with the primary antibodies (1/200 dilution ratio) indicated in Table 2. After washing with PBS, the sections were incubated again with the fluorescence-linked secondary antibodies in Table 3 (1/50 dilution ratio) for 45 min at 37°C in the dark. DAPI was dripped onto the tissue samples and covered with a coverslip and examined under a fluorescent microscope (Zeiss Axio scope).

Table 3
Fluorescence-induced secondary antibody information used in Immunofluorescence staining.

| Secondary Antibody          | Company, Catalog No.                      |
|-----------------------------|------------------------------------------|
| Goat Anti-Mouse IgG- FITC   | Jackson ImmunoResearch, Catalog No. 115-095-003 |
| Mouse Anti-Rabbit IgG-FITC  | Santa Cruz, Catalog No. sc-2359           |

Immunohistochemical and immunofluorescence positivities in 10 randomly selected areas at 20x magnification, by using the image analysis computer program named as Image J 1.43, according to the intensity of staining: none (0), mild (1), moderate (2), and severe (3).

Biochemical Analysis

Serum testosterone levels were analyzed with the trademark ELISA kit (Biovision, Catalog No: K7418) according to the kit procedure. Results are given in ng/ml for testosterone.

Histometric Calculation of Seminiferous Tubule Diameters
To measure the diameters of the seminiferous tubules, 2 measurements were made from 10 randomly selected round and near-circular seminiferous tubules from each animal at 20x magnification and their averages were taken (Mouro et al. 2018). The measurement was made using the image analysis computer program called as Image J 1.43 (Rasband 2014).

**Statistical analysis**

Non-parametric statistics were used in the study. Kruskal Wallis H Test was used to compare body weight, seminiferous tubule diameter and Johnsen Testicular Biopsy Scores between groups, and Mann Whitney U test was performed between paired groups for differences between significant variables. However, Wilcoxon Test was used for the difference in body weights of the groups on the 1st and 30th days. Analyses were carried out using SPSS v.22.

**Results**

**Live Weight Findings**

There was no statistically significant difference between the groups in terms of the weight of the rats on day 1 ($\chi^2 = 0.284, SD = 3, p > 0.05$). When the body weights of the rats were compared between the groups, a significant difference was observed ($\chi^2 = 29.856, SD = 3, p < 0.001$) at day 30. It was determined that there was no statistically significant difference between the control group and the sham group ($p = 0.171$), while the differences between all other paired groups were statistically significant ($p < 0.01$).

Control group ($z = 2.521, p < 0.05$), sham group ($z = 2.524, p < 0.05$), 0.5 mg/kg ROF ($z = 2.623, p < 0.01$) and 1 mg/kg ROF A statistically significant difference was found between the 1st and 30th day weights of the groups ($z = 2.814, p < 0.01$). When the change in body weight within 30 days was examined, a statistically significant increase at the end of 30 days in the control group and sham group; A statistically significant decrease was observed at the end of 30 days in the 0.5 mg/kg and 1 mg/kg ROF groups (Table 4).
### Table 4
Live weight values and intergroup comparisons.

| Groups                        | 1st Live Weight - mg/day mean ± SD (Min-Max) | 30th Live Weight - mg/day mean ± SD (Min-Max) | p (1st vs 30th) |
|-------------------------------|---------------------------------------------|---------------------------------------------|-----------------|
| Control (n = 8)               | 193.00 ± 5.80 (185.00–200.00)                | 210.50 ± 9.05 (197.00–220.00)               | 0.012**         |
| Sham (n = 8)                  | 192.63 ± 4.17 (187.00–200.00)                | 204.50 ± 7.70 (195.00–218.00)               | 0.012**         |
| 0.5 mg/kg ROF Group (n = 10)  | 193.80 ± 5.05 (185.00–202.00)                | 190.70 ± 4.66 (184.00–201.00)               | 0.009**         |
| 1 mg/kg ROF Group (n = 10)    | 192.80 ± 4.78 (187.00–200.00)                | 179.60 ± 2.06 (176.00–182.00)               | 0.005**         |

1st Day Live Weight Group Comparisons

| Groups                        | P               |
|-------------------------------|-----------------|
| Control – Sham                | 0.915           |
| Control – 0.5 mg/kg ROF Group | 0.859           |
| Control – 1 mg/kg ROF Group   | 1.000           |
| Sham – 0.5 mg/kg ROF Group    | 0.624           |
| Sham – 1 mg/kg ROF Group      | 1.000           |
| 0.5 mg/kg ROF Group – 1 mg/kg ROF Group | 0.595 |

30th Day Live Weight Group Comparisons

| Groups                        | P               |
|-------------------------------|-----------------|
| Control – Sham                | 0.171           |
| Control – 0.5 mg/kg ROF Group | 0.01**          |
| Control – 1 mg/kg ROF Group   | 0.000*          |
| Sham – 0.5 mg/kg ROF Group    | 0.001*          |
| Sham – 1 mg/kg ROF Group      | 0.000*          |
| 0.5 mg/kg ROF Group – 1 mg/kg ROF Group | 0.000* |
**Seminiferous Tubule Diameter**

There was a statistically significant difference between the groups in terms of seminiferous tubule diameter of rats ($\chi^2 = 23.959$, $SD = 3$, $p < 0.001$). There was no statistically significant difference between the control and sham groups in terms of seminiferous tubule diameter of rats ($p > 0.05$), but it was observed that there was statistically significant difference between the control group and 0.5 mg/kg ROF ($p < 0.05$) and 1 mg/kg ROF ($p < 0.001$) groups. However, there was no statistically significant difference between the sham group and 0.5 mg/kg ROF group ($p > 0.05$), but there was a statistically significant difference between the mg/kg ROF group and the sham group and 1 mg/kg ROF group ($p < 0.001$) and 0.5 mg/kg and 1 ($p < 0.001$) (Table 5).
Table 5
Seminiferous tubule diameter measurement results and group comparisons.

| Groups                        | Seminiferous Tubule Diameter (µm) |
|-------------------------------|-----------------------------------|
|                               | mean ± SD (Min–Max)               |
| Control (n = 8)               | 281.88 ± 1.72 (279.00–284.00)     |
| Sham (n = 8)                  | 281.00 ± 0.756 (280.00–282.00)    |
| 0.5 mg/kg ROF Group (n = 10)  | 280.20 ± 0.789 (279.00–281.00)    |
| 1 mg/kg ROF Group (n = 10)    | 275.40 ± 2.95 (271.00–279.00)     |

Seminiferous Tubule Diameter Group Comparisons

| Groups                        | P       |
|-------------------------------|---------|
| Control – Sham                | 0.197   |
| Control – 0.5 mg/kg ROF Group | 0.036** |
| Control – 1 mg/kg ROF Group   | 0.000*  |
| Sham – 0.5 mg/kg ROF Group    | 0.057   |
| Sham – 1 mg/kg ROF Group      | 0.000*  |
| 0.5 mg/kg ROF Group – 1 mg/kg ROF Group | 0.000* |

*p < 0.005, **p < 0.05, SD: Standard Deviation, ROF: Roflumilast

**Johnsen Testicular Biopsy Score**

According to the Johnsen Testicular Biopsy Score, there was no significant difference between the control and sham groups (p > 0.05), but there was a significant difference between the 0.5 mg/kg and 1 mg/kg ROF groups and the control and sham groups (p > 0.05). <0.05). Statistical analysis results according to Johnsen Testicular Biopsy Score are given in Table 6.
Table 6
Johnsen testicular biopsy results.

| Groups          | Johnsen Testicular Biopsy Score mean ± SD |
|-----------------|------------------------------------------|
| Control         | 9.50 ± 0.53a                             |
| Sham            | 9.37 ± 0.51a                             |
| 0.5 mg/kg ROF   | 7.37 ± 0.18b                             |
| 1 mg/kg ROF     | 7.50 ± 0.18b                             |

a, b Different letters in the same column show the difference between groups, SD: Standard Deviation, ROF: Roflumilast

**Histopathological Findings**

It was observed that the seminiferous tubules and spermatogenic and Sertoli cells in these tubules were normal in the testicular tissues of the rats in the control and sham groups. Interstitial connective tissue and interstitial cells were found between the seminiferous tubules. It was observed that the full structures of the seminiferous tubules began to disappear and the normal ordered structures of the spermatogenic cells began to deteriorate in the testicular tissues of the rats in the 0.5 and 1 mg/kg ROF groups. In the 0.5 mg/kg ROF group, shedding of the seminiferous epithelium and local degenerations in the interstitial area were observed, while in the 1 mg/kg ROF group, intercellular separation, desquamation, interstitial edema and degenerative changes were observed (Fig. 1).

**Immunohistochemical Findings**

Caspase-3, AIF and LC3B immunoreactivity (Fig. 2) was not statistically significant in testicular tissues of control and sham group rats (p > 0.05).

Mild Caspase-3, AIF and LC3B immunoreactivity was observed in the interstitial area in testicular tissues of rats in the 0.5 mg/kg ROF group, while mild LC3B immunoreactivity was also observed in the seminiferous epithelium (Fig. 3). Caspase-3 and AIF immunoreactivity was mild in the interstitial area, and LC3B immunoreactivity was severe in the interstitial area and seminiferous epithelium in testicular tissues of rats in the 1 mg/kg ROF group (Fig. 3). There was a statistically significant difference between the control and sham groups and the 0.5 and 1 mg/kg ROF groups (Table 7, p < 0.05).
Table 7
Statistical representation of the degree of caspase-3, AIF and LC3B immunoreactivity.

| Groups               | Caspase-3 mean ± SD | AIF mean ± SD | LC3B mean ± SD |
|----------------------|---------------------|---------------|----------------|
| Control              | 0.25 ± 0.46<sup>Aa</sup> | 0.37 ± 0.51<sup>Aa</sup> | 0.25 ± 0.46<sup>Aa</sup> |
| Sham                 | 0.12 ± 0.35<sup>Aa</sup> | 0.37 ± 0.51<sup>Aa</sup> | 0.25 ± 0.46<sup>Aa</sup> |
| 0.5 mg/kg ROF        | 1.12 ± 0.64<sup>Ba</sup> | 1.25 ± 0.70<sup>Ba</sup> | 2.12 ± 0.35<sup>Bb</sup> |
| 1 mg/kg ROF          | 2.12 ± 0.35<sup>Ca</sup> | 2.00 ± 0.53<sup>Ca</sup> | 2.87 ± 0.35<sup>Cb</sup> |

<sup>a,b,c</sup> Different letters in the same column show the difference between groups, <sup>A,B</sup> Different letters in the same line indicate the difference in the groups, SD: Standard Deviation, ROF: Roflumilast

**Immunofluorescence Findings**

It was not observed a significant immunopositivity in terms of Caspase-3, AIF and LC3B in testicular tissues of rats in the control and sham groups (Fig. 4) and no statistical difference (<i>p > 0.05</i>). It was determined that Caspase-3, AIF and LC3B expression levels in testicular tissues of rats in the 0.5 mg/kg ROF group were increased compared to the control and sham groups and were mildly severe in the interstitial area. Caspase-3, AIF and LC3B immunopositivity in testicular tissues of rats in the 1mg/kg ROF group was moderate in the interstitial area (Fig. 5). There was a significant difference between the control and sham groups and the 0.5 and 1 mg/kg ROF groups (Table 8, <i>p < 0.05</i>).
Table 8
Statistical representation of immunofluorescence staining in terms of caspase-3, AIF and LC3B.

| Groups           | Caspase-3 mean ± SD | AIF mean ± SD  | LC3B mean ± SD |
|------------------|---------------------|----------------|----------------|
| Control          | 0.50 ± 0.53Ã±       | 0.37 ± 0.51Ã±  | 0.50 ± 0.53Ã±  |
| Sham             | 0.62 ± 0.51Ã±       | 0.50 ± 0.53Ã±  | 0.75 ± 0.46Ã±  |
| 0.5 mg/kg ROF    | 1.75 ± 0.46Bb       | 1.25 ± 0.70Bb  | 1.87 ± 0.35Bb  |
| 1 mg/kg ROF      | 1.87 ± 0.35Bb       | 1.75 ± 0.46Bb  | 2.00 ± 0.53Bb  |

a,b Different letters in the same column show the difference between groups, A,B Different letters in the same line indicate the difference in the groups, SD: Standard Deviation, ROF: Roumilast

Biochemical Findings

In terms of serum testosterone levels, there was no statistically significant difference between the control and sham groups (p = 0.144), the 0.5 mg/kg ROF group was lower than the control and sham groups (p = 0.001 and p = 0.014, respectively), and the 1 mg/kg ROF group was found to be lower compared with the control, sham and 0.5 mg/kg ROF groups (p = 0.000, p = 0.000, p = 0.027, respectively) (Table 9).

Table 9
Serum testosterone level result.

| Groups           | Serum Testosterone Level (ng/mL) mean ± SD |
|------------------|--------------------------------------------|
| Control          | 0.48 ± 0.01 a                             |
| Sham             | 0.46 ± 0.02 a                             |
| 0.5 mg/kg ROF    | 0.40 ± 0.05 b                             |
| 1 mg/kg ROF      | 0.35 ± 0.03 b                             |

a,b Different letters in the same column show the difference between groups, SD: Standard Deviation, ROF: Roumilast

Ethics
The approval was obtained by the Animal Experiments Local Ethics Committee of Atatürk University, Faculty of Veterinary Medicine, on the date of 31.07.2019 with the ethics committee number of 9/130 for this study. The rats used in the study were obtained from Atatürk University Medical Experimental Research and Application Center and the experimental part of the study was conducted in this center. The research was carried out following the principles of the Declaration of Helsinki.

**Discussion**

Chronic bronchitis symptoms of Roumilast and are the only PDE4 inhibitor for COPD patients with a history of exacerbation that has reached the market (Baye 2012). Several new PDE4 inhibitor compounds are in early clinical development, and there is not yet clear information about their efficacy and safety (Sugin et al. 2020). In preclinical and early-stage studies, it has been reported that PDE4 inhibitors improve memory and have curative effects on depressive-like behaviors caused by chronic mild stress, lipopolysaccharide or ethanol abstinence (Prickaerts, Heckman, and Blokland 2017; Yu et al. 2018). Roumilast has been studied by many researchers to determine the mechanism and new indications. Wang et al. were applied orally 5–10 mg/kg Roumilast once a day for 30 days, and it was determined that it had anti-apoptotic and anti-inflammatory effects on nerve cells. It has also been reported that it can be an effective agent in memory impairment and in the treatment of depression (Wang et al. 2020). In cadmium (Cd)-derived nephrotoxic rats, they reported a significant increase in anti-oxidative enzymes such as Superoxide Dismutase, Catalase and Glutathione-S-transferase, and a decrease in oxidative parameters such as Malondialdehyde and ischemic modified albumin. However, it has been reported that the damage caused by Cd in kidney tissues is significantly recurred of Roumilast treatment (0.5 and 1.5 mg/kg) (Ansari et al. 2019).

It has been asserted that Roumilast, which is used orally once a day, does not cause arrhythmia and the most common side effects are diarrhea and weight loss (AYDEMİR 2018). It has been reported that weight loss, one of the most important side effects of Roumilast, was observed in 83 adult COPD cases because of treatment with Roumilast (500 µg tablet, 18 months, once a day) (Cilli, Bal, and Gunen 2019). We found that while an increase in rat body weight was observed in the control and sham groups in accordance with the months, the body weights of the rats exposed to Roumilast decreased as the dose increased in accordance with the literature.

It has been reported that the dose and duration of application of phosphodiesterase inhibitors are determinative for efficacy, but long-term use at high doses causes adverse effects (Kızılay and Altay). In our study, in the 0.5 mg/kg Roumilast group, the testicular tissue of the rats was narrowed in seminiferous tubule diameter and degeneration was observed in the interstitial area, while the damage become clearer in the 1 mg/kg Roumilast group. It was observed that the narrowing of the tubule diameter was statistically significant, separation between cells, desquamation, degenerative changes and edematous areas in the interstitial connective tissue.
It has been reported that PDE4 increases cell viability and mitochondrial activity, and decreases cell death (Wedzicha, Calverley, and Rabe 2016; Kyung et al. 2018). It has been asserted that PDE4 may be a new therapeutic agent in liver fibrosis, increasing the level of cAMP, which suppresses collagen synthesis and fibroblast activation (Essam et al. 2019). The effects of Roflumilast on cerebral inflammation developing in the subarachnoid hemorrhage model in rats were investigated and it was shown that it significantly reduced neurological damage and inflammatory cytokines IL1β, IL-6 and TNFα levels and the number of apoptotic neurons (Wu et al. 2017). As a result of examining the cerebral cortices of rats applied Roflumilast by TUNEL method, it was determined that apoptosis was significantly reduced (Wang et al. 2020). In a study in which a sepsis model was created by cecal ligation and puncture surgery, Roflumilast (1 mg/kg and 3 mg/kg) was applied to mice once a day for 7 days, and it was concluded that 3 mg/kg Roflumilast could protect mice against kidney damage by partially reducing cell apoptosis (Xu et al. 2020). In the study, in which the PDE4 enzyme inhibitor rolipram was applied to rats (single dose 10 mg/kg) 15 minutes before detorsion and the effects on histopathological damage after testicular torsion/detorsion and the apoptotic pathways thought to cause damage; It was found that rolipram could not reverse histological damage, suppress the activated intrinsic apoptotic pathway, and did not have a protective effect against histopathological damage (Akdeniz E 2018.). We determined that caspase-3 expression increased in healthy testicular tissue, especially at high doses, in chronic use of Roflumilast in line with the results of this study.

It is emphasized that in male reproduction of cAMP, while it is reported that the improvements of sperm motility with hyperactivity of the ability to enter the changes and the acrosome reaction and developments during the capacitation process is required, in case of increasing of cAMP levels in an uncontrollable way also may cause hyper-proliferation in the cellular processes including cancer development can cause problems in cellular process with many studies (Aitken and De Iuliis 2009; Yan et al. 2016). It has been stated that while apoptosis that occurs under physiological conditions in the immature testis is necessary for the development of germ cells, misactivation of apoptosis may impair spermatogenesis and cause reproductive defects (Kumar, Abbas, and Aster 2013; Qian et al. 2020). In the results of this study, it was observed that the intensity of Caspase-3 and AIF immunoreactivity, which are important apoptosis reagents in spermatogenesis, increased more in rat testis tissue in 1 mg/kg Roflumilast group compared to 0.5 mg/kg Roflumilast group. Researchings related on the subject show that chronic and high doses use of Roflumilast may affect the spermatogenesis process negatively.

Autophagy activation has been shown to play a regulatory role in the maintenance of spermatogenesis and the maintenance of spermatogenic stem cells. When the activation of LC3-2, LC3-2/LC3-1, Atg5 and Beclin-1 autophagic reagents were examined by spermatogenic stem cell culture study of organophosphate, which is toxic for the reproductive system; it has been reported that all these reagents are increased in rat spermatogenic stem cells (Liu et al. 2014). In the study investigating the role of autophagy in testicular tissue, germ cell specific Atg7 deletion was performed and the fertility of Atg7 mice was evaluated. It has been observed that there are very few sperms in the epididymis, most of the sperms exhibit round head anomaly, and the acrosome structure is damaged (Wang et al. 2014). In this study, we found that LC3B expression increased in rats exposed to 0.5 and 1 mg/kg Roflumilast for 4
weeks, and there was positivity in some spermatogenic cells, unlike caspase-3 and AIF. Based on these findings, it was thought that structural and functional changes in testicular tissue might be associated with increased autophagy.

Testosterone is an important androgen in the development of the male reproductive system and sexual characteristics. It has been reported that testosterone regulates the cGMP pathway and thus affects endothelial function and endothelial progenitor cells, which are key to the endothelial repair system (Zgair et al. 2021). It has been asserted that decreased testosterone level causes structural and functional changes in Sertoli cells, which are important for germ cells (Dym and Madhwa Raj 1977). After long-term treatment with sildenafil, a PDE5 inhibitor; it has been reported that the circulating testosterone level is normalized and contributes to the accumulation of cGMP in the testicular interstitial fluid, resulting as an improvement in Leydig cell steroidogenic capacity and an increase in the number of Leydig cells, in addition to preventing the atrophy of the seminiferous tubules (Sokanovic et al. 2018). The inhibition of PDE4 enzymes is in testosterone production by the Leydig cells have been reported to cause a significant increase. In rats, it has been asserted that autophagy participates in the regulation of steroid synthesis in Leydig cells (Weckmann et al. 2018). It has been reported that PDE4 and PDE8 play important roles in the physiology of steroidogenic tissues and there is synergism between signaling compartments regulated by PDE4 and PDE8 to facilitate maximum steroid output (Golkowski et al. 2016). It has been asserted that the repository of cAMP, which regulates androgen production, is controlled by PDE8s working together with PDE4, and that PDE inhibitor therapy should simultaneously target both PDE8 isozymes and PDE4 to be an effective stimulator of steroidogenesis (Shimizu-Albergine et al. 2016). In our findings, it was revealed by ELISA method that the serum testosterone level was within the normal reference range in the control and sham groups (p = 0.001 and p = 0.014), but with the increase in the dose used in the Roflumilast groups, the testosterone level decreased statistically significantly (p = 0.000, p = 0.000, p = 0.027).

As a result, it is considered that degenerated and narrowed seminiferous tubule structures, increased caspase-3, AIF and LC3B immunoreactivity density, decrease in serum testosterone level with increasing dose; Roflumilast may have some anabolic effects and have negative effects on spermatogenesis and therefore male fertility due to changes in the mechanism of apoptosis and autophagy with this study. However, more detailed experimental studies and clinical findings are needed to determine the toxicities that may occur in chronic use of high doses of Roflumilast.

Declarations

Author Contributions: AG and EKS: Conceptualization, data curation, investigation, formal analysis, original draft preparation, pharmacokinetics and tissue distribution of Roflumilast in rats, methodology and writing, editing and review. All authors have published version of the manuscript and agreed to the read.

Disclosure of Interest
Authors of this manuscript have no conflict of interest to declare.

References

Aitken RJ, De Iuliis GN (2009) 'On the possible origins of DNA damage in human spermatozoa'. MHR: Basic science of reproductive medicine 16:3–13

Akdeniz E, Dengiz G, Yılmaz Z (2018) "Fosfodiesteraz 4 Enzim İnhibitörü Rolipramın Sıçanlarda Testiküler İskemi Reperfüzyon Hasarı Üzerine Etkileri." In. Bülent Ecevit Üniversitesi/ Sağlık Bilimleri Enstitüsü

Ansari MN, Aloliet RI, Ganaie MA, Khan TH, Najeeb-ur-Rehman F, Imam, Hamad AM (2019) 'Roumilast, a phosphodiesterase 4 inhibitor, attenuates cadmium-induced renal toxicity via modulation of NF-κB activation and induction of NQO1 in rats', Human & experimental toxicology, 38: 588 – 97

AYDEMİR Y (2018) 'Asthm, Kronik Obstrüktif Akciğer Hastalığı ve Fibrozis Hastalıklarının Karşılaştırılması Klinik Farmakolojisi'. Turkiye Klinikleri J Pharmacol-Special Topics 6:118–125

Bajpai VK, Imran Khan S, Shukla S-M, Kang F, Aziz KM, Tripathi D, Saini H-J, Cho NS, Heo, Sumit KS (2020) 'Multifunctional NP-doped carbon dots for regulation of apoptosis and autophagy in B16F10 melanoma cancer cells and in vitro imaging applications', Theranostics, 10: 7841

Baye J (2012) 'Roflumilast (dairesp): a novel phosphodiesterase-4 inhibitor for the treatment of severe chronic obstructive pulmonary disease'. Pharmacy Therapeutics 37:149

Botros SS, Naglaa M, El-Lakkany SHS, El-Din S, William A-N, Sabra, Olfat A, Hammam, Harry P, de Koning (2020) 'The phosphodiesterase-4 inhibitor roflumilast impacts Schistosoma mansoni ovipositing in vitro but displays only modest antischistosomal activity in vivo'. Experimental parasitology 208:107793

Cilli A, Bal H, Hakan Gunen (2019) 'Efficacy and safety profile of roflumilast in a real-world experience', Journal of thoracic disease, 11: 1100

Drobnis EZ, Ajay KN (2017) 'Phosphodiesterase inhibitors (PDE inhibitors) and male reproduction', Impacts of Medications on Male Fertility: 29–38

Dym M, Madhwa Raj HG (1977) 'Response of adult rat Sertoli cells and Leydig cells to depletion of luteinizing hormone and testosterone', Biology of reproduction, 17: 676 – 96

Erboga M, Kanter M, Aktas C, Donmez YB, Erboga ZF, Aktas E, Ahmet Gurel (2016) 'Anti-apoptotic and anti-oxidant effects of caffeic acid phenethyl ester on cadmium-induced testicular toxicity in rats', Biol Trace Elem Res, 171: 176–184

Essam RM, Lamiaa A, Ahmed, Rania M, Abdelsalam, El-Khatib AS (2019) 'Phosphodiesterase-1 and 4 inhibitors ameliorate liver fibrosis in rats: Modulation of cAMP/CREB/TLR4 inflammatory and fibrogenic pathways', Life sciences, 222: 245 – 54
Fala L (2015) 'Otezla (Apremilast), an oral PDE-4 inhibitor, receives FDA approval for the treatment of patients with active psoriatic arthritis and plaque psoriasis'. American health drug benefits 8:105

FDA (2011) 'Center for drug evaluation and research', Accessed 2021 May 31. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/022522Orig1s000PharmR.pdf

Fleming P, Yang YB, Lynde C, BO'Neill, Kyle OL (2020) 'Diagnosis and management of atopic dermatitis for primary care providers'. The Journal of the American Board of Family Medicine 33:626–635

García-Prat L, Martínez-Vicente M, Perdiguero E, Ortet L, Antonio LS (2016) Javier Rodríguez-Ubreva, Elena Rebollo, Vanessa Ruiz-Bonilla, Susana Gutarra, Esteban Ballestar, and. Nature 529:37–42 'Autophagy maintains stemness by preventing senescence'

Giembycz MA, Stephen KF (2010) 'Roflumilast: first phosphodiesterase 4 inhibitor approved for treatment of COPD', Drug design, development and therapy, 4: 147

Golkowski M, Shimizu-Albergine M, Suh HW, Beavo JA, Shao-En O (2016) 'Studying mechanisms of cAMP and cyclic nucleotide phosphodiesterase signaling in Leydig cell function with phosphoproteomics', Cellular signalling, 28: 764 – 78

Hatzelmann A, Morcillo EJ, Lungarella G, Adnot S, Sanjar S, Beume R, Schudt C, Hermann Tenor (2010) 'The preclinical pharmacology of roflumilast—a selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease', Pulm Pharmacol Ther, 23: 235–256

Herb M, Gluschko A, Schramm M (2020) "LC3-associated phagocytosis-The highway to hell for phagocytosed microbes. In: " In Seminars in cell & developmental biology. Elsevier, pp 68–76

Johnsen SG (1970) 'Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males'. Hormone Research in Paediatrics 1:2–25

Kızılay, Fuat, and Barış Altay. 'Fosfodiesteraz Tip-5 inhibitörlerinin semen parametrelerine etkisi'

Kumar V, Abbas AK, Jon CA (2013) 'Cell injury, cell death, and adaptations'. Robbins basic pathology 8:1–30

Kyung S, Young YJ, Kim ES, Son SH, Jeong, Jeong-Woong Park (2018) 'The phosphodiesterase 4 inhibitor roflumilast protects against cigarette smoke extract-induced mitophagy-dependent cell death in epithelial cells'. Tuberc Respir Dis 81:138–147

Liu M-L, Wang J-L, Wei J, Xu L-L, Yu M, Liu X-M, Wen-Li Ruan, and Jia-Xiang Chen. 2014. 'Tri-ortho-cresyl phosphate induces autophagy of rat spermatogonial stem cells', Reproduction (Cambridge, England), 149: 163–70
Mouro, Viviane GS, Tatiana P, Menezes GDA, Lima RR, Domingues, Ana Cláudia F, Souza JA, Oliveira, Sérgio LP Matta, and Mariana Machado-Neves. 2018. 'How bad is aluminum exposure to reproductive parameters in rats?', Biol Trace Elem Res, 183: 314–324

Murad HA, Hamed S, Habib MM, Rafieeq MI, Sulaiman AS, Abdulrahman, Mohamad Nidal K (2017) 'Co-inhalation of roflumilast, rather than formoterol, with fluticasone more effectively improves asthma in asthmatic mice'. Experimental Biology Medicine 242:516–526

Nabavi S, Mohammad S, Talarek J, Listos SF, Nabavi KP, Devi, Marcos Roberto de Oliveira, Devesh Tewari, Sandro Argüelles, Saeed Mehrzadi, and Azam Hosseinzadeh. 2019. 'Phosphodiesterase inhibitors say NO to Alzheimer's disease', Food and Chemical Toxicology, 134: 110822

Ouyang P, Feng Y, Xiong G, Liu R, Wei F, Wang K, Geng Y, Huang X, Chen D, Shiyong Yang (2021) 'Potential mechanism of the PDE-cAMP-related network action on hepatopancreatic necrosis syndrome of Chinese mitten crab (Eriocheir sinensis)', Aquaculture, 531: 735982

Prickaerts J, Pim RA, Heckman, Blokland A (2017) 'Investigational phosphodiesterase inhibitors in phase I and phase II clinical trials for Alzheimer's disease'. Expert opinion on investigational drugs 26:1033–1048

Qian Y-C, Xie Y-X, Wang C-S, Shi Z-M, Jiang C-F, Tang Y-Y, Qian Xu, Wang L, Bing-Hua J (2020) 'Mkm2 deficiency induces teratozoospermia and male infertility through p53/PERP-mediated apoptosis in testis'. Asian journal of andrology 22:414

Rasband WS (2014) 'ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, imagej. nih.gov/ij/, 1997–2012', JMP®, Versión, 7: 1989–2007

Shimizu-Albergine M, Van Yserloo B, Golkowski MG, Ong S-E, Beavo JA, Karin EB (2016) 'SCAP/SREBP pathway is required for the full steroidogenic response to cyclic AMP', Proceedings of the National Academy of Sciences, 113: E5685-E93

Sokanovic SJ, Ivan Capo, Marija M, Medar SA, Andric, Tatjana SK (2018) 'Long-term inhibition of PDE5 ameliorates aging-induced changes in rat testis', Experimental gerontology, 108: 139 – 48

Sugin L, Jabaris S, Arumugam Murugesan M, Bindu, Narayan Sunil K (2020) 'Roflumilast: A potential drug for the treatment of cognitive impairment?', Neuroscience Letters: 135281

Voss AK, and Andreas Strasser (2020) 'The essentials of developmental apoptosis', F1000Research, 9

Wang H, Zhang Fang-fang, Xu Y, Fu Hua-rong, Wang Xiao-dan, Wang L, Chen W, Xu Xiao-yan, Gao Yong-feng, Ji-guo Z (2020) 'The phosphodiesterase-4 inhibitor roflumilast, a potential treatment for the comorbidity of memory loss and depression in Alzheimer's disease: A preclinical study in APP/PS1 transgenic mice'. Int J Neuropsychopharmacol 23:700–711
Wang Y, Zheng W, Bian X, Yuan Y, Gu J, Liu X, Liu Z, Jianchun Bian (2014) 'Zearalenone induces apoptosis and cytoprotective autophagy in primary Leydig cells', Toxicology letters, 226: 182–191

Weckmann K, Diefenthäler P, Baeken MW, Yusifli K, Christoph W, Turck JM, Asara C, Behl, Parvana Hajieva (2018) 'Metabolomics profiling reveals differential adaptation of major energy metabolism pathways associated with autophagy upon oxygen and glucose reduction', Scientific reports, 8: 1–14

Wedzicha JA (2013) 'Dual PDE 3/4 inhibition: a novel approach to airway disease?', The Lancet. Respiratory Medicine, 1: 669 – 70

Wedzicha JA, Peter MA, Calverley, Klaus FR (2016) 'Roflumilast: a review of its use in the treatment of COPD'. Int J Chronic Obstr Pulm Dis 11:81

Wu Q, Qi L, Li H, Mao L, Yang M, Xie R, Yang X, Wang J, Zhang Z, Jiming Kong (2017) 'Roflumilast reduces cerebral inflammation in a rat model of experimental subarachnoid hemorrhage', Inflammation, 40: 1245–1253

Xu X, Liao L, Hu B, Jiang H, Meichun, Tan (2020) 'Roflumilast, a Phosphodiesterases-4 (PDE4) Inhibitor, Alleviates Sepsis-induced Acute Kidney Injury'. Medical Science Monitor: International Medical Journal of Experimental Clinical Research 26:e921319–e921311

Yan KUO, Li–Na Gao Yuan–Lu, Cui YI, Zhang, Zhou XIN (2016) 'The cyclic AMP signaling pathway: Exploring targets for successful drug discovery'. Mol Med Rep 13:3715–3723

Yu H, Zou Z, Zhang X, Peng W, Chen C, Ye Y, Xu J, Wang H (2018) 'Inhibition of phosphodiesterase 4 by FCPR03 alleviates lipopolysaccharide-induced depressive-like behaviors in mice: involvement of p38 and JNK signaling pathways'. Int J Mol Sci 19:513

Yu Q, Lu Z, Tao L, Yang Lu, Guo Yu, Yang Y, Xude Sun, and Ding Q (2015) 'ROS-dependent neuroprotective effects of NaHS in ischemia brain injury involves the PARP/AIF pathway'. Cellular physiology biochemistry 36:1539–1551

Zgair A, Dawood Y, Ibrahim SM, Lee JB, Feng W, Peter M, Fischer, Pavel Gershkovich (2021) 'Strawberry decreases intraluminal and intestinal wall hydrolysis of testosterone undecanoate', Molecules, 26: 233

Zheng C-X, Lu M, Guo Y-B, Zhang F-X, Liu H, Guo F, Huang X-L, Xiao-Hua Han (2016) 'Electroacupuncture ameliorates learning and memory and improves synaptic plasticity via activation of the PKA/CREB signaling pathway in cerebral hypoperfusion', Evidence-Based Complementary and Alternative Medicine, 2016

Figures
Figure 1

Rat testicular tissue, H&E staining. (a): Sham group, (b): 0.5 mg/kg ROF group, (c): 1 mg/kg ROF group, Seminiferous epithelium (se), Degenerate tubule (*), interstitial edema (○), x40-H&E.

Figure 2
Figure 3

Rat testicular tissue, Immunohistochemical staining. (a): Control group Caspase-3 negativity, (b): Control group AIF negativity, (c): Control group LC3B negativity, (d): Sham group Caspase-3 negativity, (e): Sham group AIF negativity, (f): Sham group LC3B negativity, x20-IHC.

Rat testicular tissue, Immunohistochemical staining. (a): 0.5 mg/kg ROF group Caspase-3 positivity, (b): 0.5 mg/kg ROF group AIF positivity, (c): 0.5 mg/kg ROF group LC3B positivity, (d): 1 mg/kg ROF group Caspase-3 positivity, (e): 1 mg/kg ROF group AIF positivity, (f): 1 mg/kg ROF group LC3B positivity Interstitial area (\[\text{\textbullet}\]), x20-IHC.
Figure 4

Rat testicular tissue, immunofluorescence staining. (a): Control group Caspase-3 immunoreactivities, (b): Control group AIF immunoreactivities, (c): Control group LC3B immunoreactivities, (d): Sham group Caspase-3 immunoreactivities, (e): Sham group AIF immunoreactivities, (f): Sham group LC3B immunoreactivities, x20-IF.
Figure 5

Rat testicular tissue, immunofluorescence staining. (a): 0.5 mg/kg ROF group, Caspase-3 immunoreactivities, (b): 0.5 mg/kg ROF group, AIF immunoreactivities, (c): 0.5 mg/kg ROF group, LC3B immunoreactivities, (d): 1 mg/kg ROF group, Caspase-3 immunoreactivities, (e): 1 mg/kg ROF group, AIF immunoreactivities, (f): 1 mg/kg ROF group, LC3B immunoreactivities. Interstitial area (○), x20-IF.