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Immunohistochemistry Study of Pentoxifylline in the Treatment of Alopecia Induced by Cyclophosphamide in Male Rats

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***Abstract*---**Objective: Hair is the human body's major esthetic display component, especially in cultural and social interactions. Alopecia starts to be diagnosed medically at a level that about 25 to 40 percent of the person's hair is dropped. Throughout this research, the potency of topical pentoxifylline in stimulating regrowth in hair loss caused by cyclophosphamide was established. Methods: The study was performed by treatment with pentoxifylline topically in male Swiss albino rats. The animals divided into seven groups with six animals in each group. To induce alopecia Cyclophosphamide used at a dose of (125 mg/kg) intraperitoneal. The animals in this study have hair clippered at day one and at day six of experiment to induce entrance of hair follicles into anagen phase. All groups injected cyclophosphamide at day 15 of experiment except control group injected only distilled water. The treatment period was fourteen days for rats used in this study. All animals in groups of study sacrificed at day 29 of experiment. Results: Hair weight assessment showed improvement in hair growth with increasing pentoxifylline concentration in groups. Strong follicular proliferation was demonstrated by histopathology and gross morphological observations of hair growth in shaved areas. Follicles count increased as well as increment of anagen percent by action of pentoxifylline. Conclusion: Topical administration of pentoxifylline 3% induced hair growth in cyclophosphamide induced alopecia.

***Keywords*---**cyclophosphamide, pentoxifylline, alopecia.

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Introduction

Hair has several beneficial vital functions, including defense from contaminants and sweat-gland dispersion substances e.g. pheromones(1). Alopecia is defined as the scalp hair loss influencing many men throughout their entire life and around 30 % of women. Alopecia starts to be diagnosed medically at a level that about 25-40 % of the person's hair is dropped.

Hair shaft comprises of three main elements which are a complex system. The medulla is covered by cortex and cuticle that comprises the innermost component. The hair fiber comes from the follicle in the scalp, which when seen in the longitudinal segment consists of several major components(2).

Hair cycle

The hair follicle inter through growth cycles. Hair follicles experience continuous cycles begin with growing phases (anagen), and then inter a decline (catagen), then a phase of resting (telogen). Around 80% of hair follicles are within the vital stage of growth. Through anagen phase, the active matrix cells start to differentiate and divide into the hair bulb. The result is a daily hair growth. Around 5-10% of HFs are in the phase of telogen, whereby all mitosis is halted. The remainder 1 to 3% is in the Catagen phase(3,4).

The hair when splitted from the follicle, it becomes the last stage in the hair process exogen. For every 100,000 hairs are measured in a healthy scalp approximately 125 hairs are damaged in the daily hair process. This hair shed normally happens during washing and hair combing, and a smaller proportion of hair dropping in each case can be found in patients who wash their hair less regularly(3,5).

Epidemiology of alopecia

The incidence estimates of alopecia as stated by Ludwig's classification scale is significantly lower in women, however after the menopause alopecia is more prevalent(6). Earlier research stated the occurrence of alopecia ranges among people, becoming prevalently within white races, which contrasts well with a recent clinical-based survey showing lesser alopecia prevalence among Korean males 14% and females 6%(7–9). From the other side, the prevalence of alopecia among people, other than Caucasian races, is 30-40 % as stated by those most scientific studies(10,11), which vary from that of the preceding studies.

Cyclophosphamide

Cyclophosphamide administration affects the ability of the heart mitochondria to retain accumulated calcium. Calcium leak from sarcoplasmic reticulum can lead to mitochondrial calcium overload, leading to reduced production of adenosine triphosphate and increased release of reactive oxygen species.

Pentoxifylline (PTX)

Pentoxifylline exhibit many actions and used for many conditions. The physicochemical characteristics of topical PTX(12) including percutaneous absorption(13,14), analgesic(15), anti-inflammatory(16) and anti-ischemic(17) effects have been evaluated(18).
The apparent first-pass impact leads to poor bioavailability of oral PTX about 20% and a half-life of 0.4-0.8 h. In addition, both oral and intravenous treatments can nearly have the same degree side consequences, including the more severe cases of sicchasia, dizziness, vomiting, and losing appetite. PTX is also best adapted for potential usage in tropical diseases(13,19).

Pentoxifylline has played a part in the treatment of a variety of skin diseases. One of most popular ones is peripheral vascular diseases. Several trials have demonstrated the clinical effectiveness of PTX in a great number of individuals with peripheral vascular disorder (2021). Nevertheless, the primary recommendation for use of intermittent claudication remains(22). Intravenous PTX may be beneficial in acute ischemic lesions of systemic sclerosis(23).

Aim and objective

- Assessment of hair growth after treatment with topical application of pentoxifylline.
- Evaluate pentoxifylline effect on hair follicles.

Material and Methods
Animal selection

Wistar Albino male rats were used in the current study, their weight ranges 120-140 gram. The animals maintained on a normal condition of humidity, temperature and the light/dark cycle. Forty two animals were selected and fed standard rodent pellet diet and they have free access to water. The animals in current study divided into seven groups, every group with six animals.

The protocol started by induction of hair stages into anagen by clippering hair in the dorsal area of animal. At the day nine all animals injected a single intraperitoneal CYP at a dose of 125mg/ml except negative control group injected distilled water. At day fifteen of study the treatment started.

- The first group assigned as negative control and didn't receive treatment.
- The second group assigned as positive control and didn't receive treatment.
- The third group assigned as PTX 1% and applied PTX at concentration of 1% topically for fourteen days.
- The fourth group assigned as PTX 2% and applied PTX at concentration of 2% topically for fourteen days.
- The fifth group assigned as PTX 3% and applied PTX at concentration of 3% topically for fourteen days.
- The sixth group assigned as vehicle and applied HPMC gel only topically for fourteen days.

- The seventh group assigned as standard and applied minoxidil at concentration of 2% topically for fourteen days.

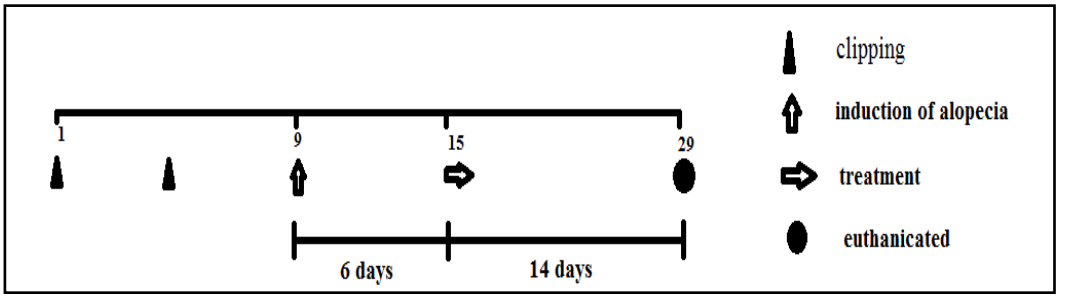

Figure 1. The protocol used for study

Histological process

Approximated regions of mice dorsal skin tissue have been sampled and put in 10% neutral buffered formalin, all dorsal skin tissues were re-fixed again for 24 hours. Three to four mm sections were prepared after paraffin embedding. For general histological architecture, descriptive parts are stained with hematoxylin and eosin (H&E). Under a light microscope, the histological profiles of individual cross-cut dorsal skin tissues were observed. The histopathological study was performed by the histopathologist, who was not aware of the group distribution(24).

Anagen percent calculation

Anagen HFs and total count of hair follicles measured per mm^2 of dorsal skin area(25). Percent of anagen was calculated using the following equation:
Anagen percent = (number of anagen HFs/total follicle count) *100%

Statistical analysis

By using Statistical Package for Social Science (SPSS 21 IBM, Armonk, USA), The results were expressed as mean ± standard deviation (SD) and were analyzed by analysis of variance (one way ANOVA) test. P value that is lower than 0.05 were considered as a statistically significant for all data(26).

Results
Weight of the hair for 3cm2 of skin dorsal area of the animals

Analysis of variance (ANOVA) expressed significant decrease ($P < 0.05$) in mean hair weight as compared PTX treated groups (1%, 2% and 3%) with standard group. In addition there is significant increase ($P < 0.05$) in hair weight level when compared PTX treated groups (1%, 2% and 3%) with positive control group (table 1).

Table 1
Effect of PTX on mean hair weight for the seven groups in the study

Group	Hair weight in mg
Negative control	0.08533±0 .009812
Positive control (Cyclophosphamide)	0.02567±0.006532
PTX 1%	0.03317±0.004535
PTX 2%	0.03900±0.003286
PTX 3%	0.04517±0.002317
Standard	0.06200±0.005762
Vehicle	0.02533±0.002733

Data are expressed as mean ± S.D; n=6 animals in each group

Effect of PTX on number of follicle per mm²

Analysis of variance (ANOVA) expressed significant decrease ($P < 0.05$) in mean number of follicles as compared PTX treated groups (1%, 2% and 3%) with standard group. In addition there is significant increase ($P < 0.05$) in number of follicles level when compared PTX treated groups (1%, 2% and 3%) with positive control group (table 2).

Table 2
Effect of PTX on number of follicles per mm²

Group	Number of follicle per mm²
Negative control	37.50± 1.049
Positive control (Cyclophosphamide)	13.67± 1.506
PTX 1%	66.83± 1.472
PTX 2%	103.33± 1.211
PTX 3%	198.33± 2.16
Standard	109.50± 1.871
Vehicle	11.17± 1.161

Data are expressed as mean ± S.D; n=6 animals in each group

Effect of PTX on anagen percent in CYP induced alopecia

Analysis of variance (ANOVA) expressed significant decrease ($P < 0.05$) in mean anagen percent as compared PTX treated groups (1%, 2% and 3%) with standard group. In addition there is significant increase ($P < 0.05$) in anagen percent level when compared PTX treated groups (1%, 2% and 3%) with positive control group (table 3).

Table 3
Effect of PTX on anagen percent in CYP induced alopecia

Group	Anagen percent
Negative control	29.5± 1.04881
Positive control (Cyclophosphamide)	8.5± 0.83666
PTX 1%	19.1667± 0.98319

PTX 2%	43.3333± 1.03280
PTX 3%	63.3333± 1.21106
Standard	55.4167± 1.02062
Vehicle	18.1667± 0.40825

Data are expressed as mean±S.D; n=6 animals in each group

Histopathology

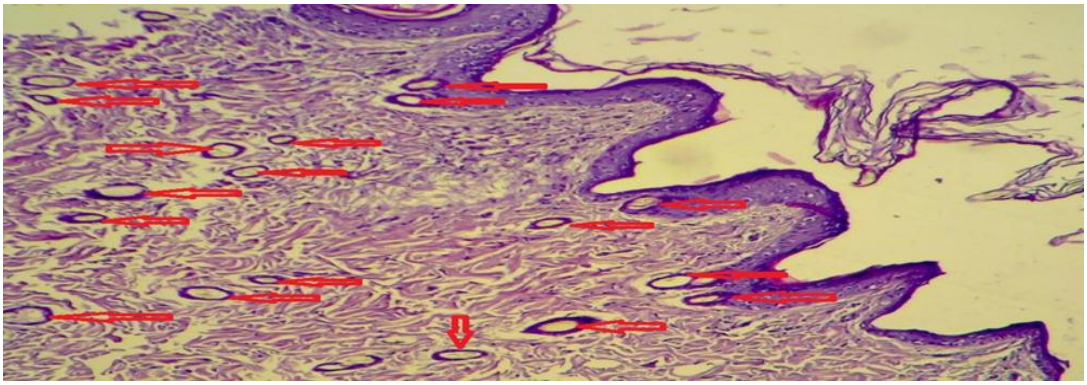

Figure 2. Negative control group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

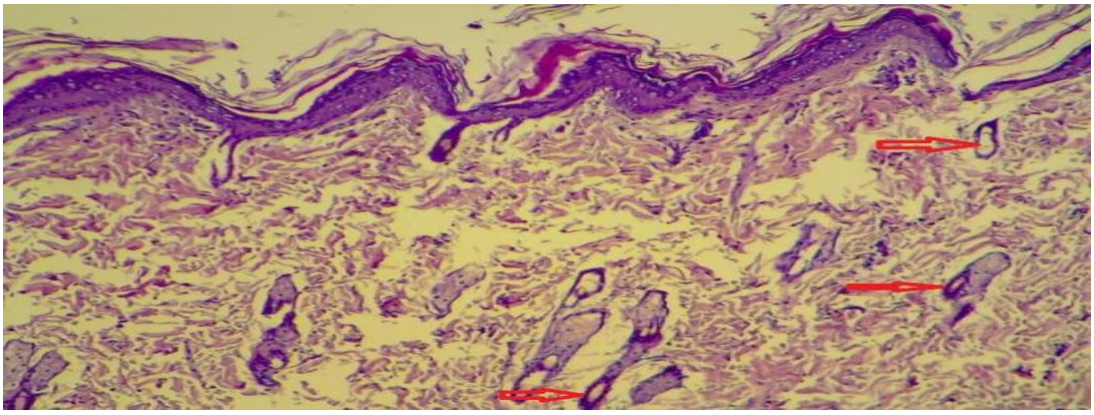

Figure 3. Positive control (Cyclophosphamide) Group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

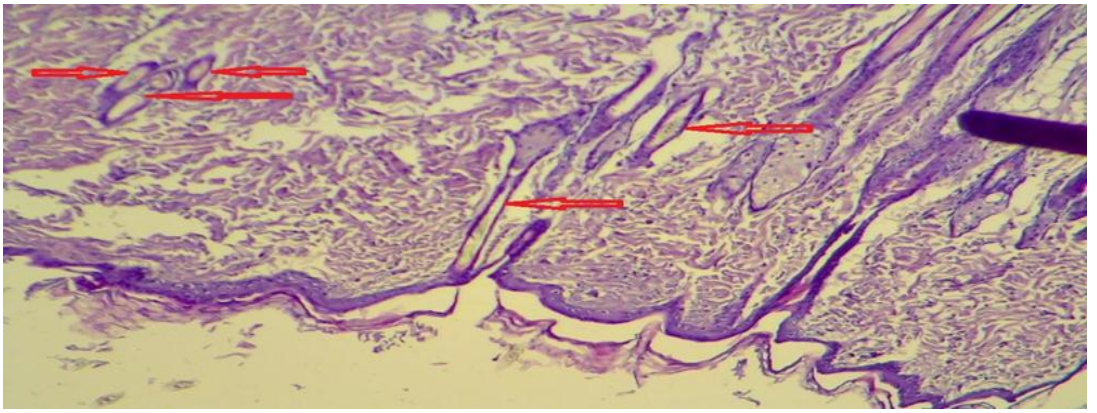

Figure 4. Pentoxifylline 1% group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

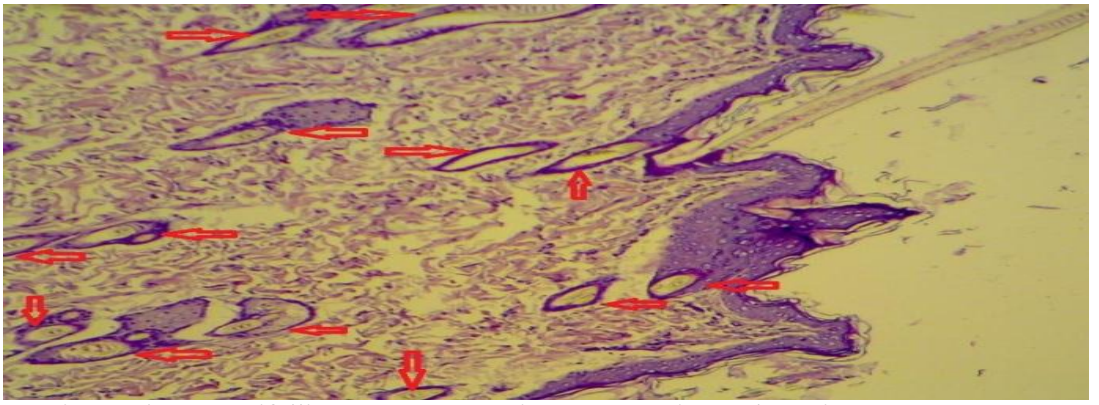

Figure 5. Pentoxifylline 2% group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

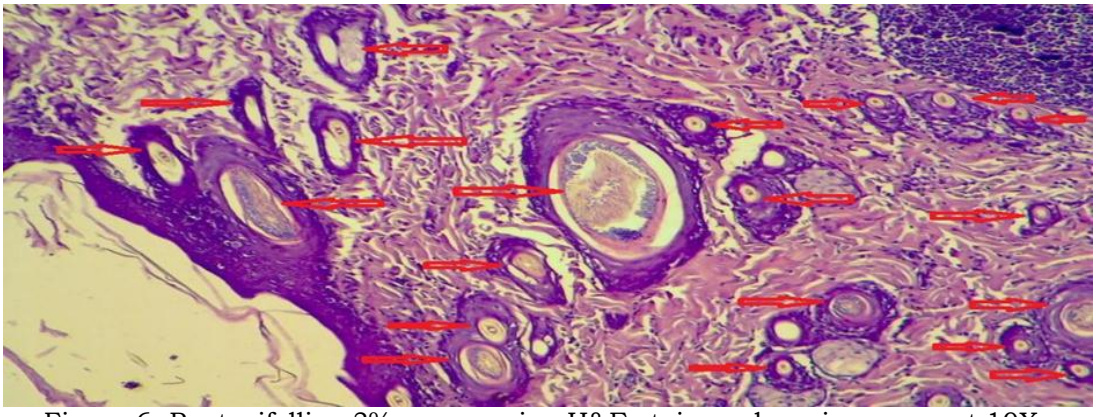

Figure 6. Pentoxifylline 3% group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

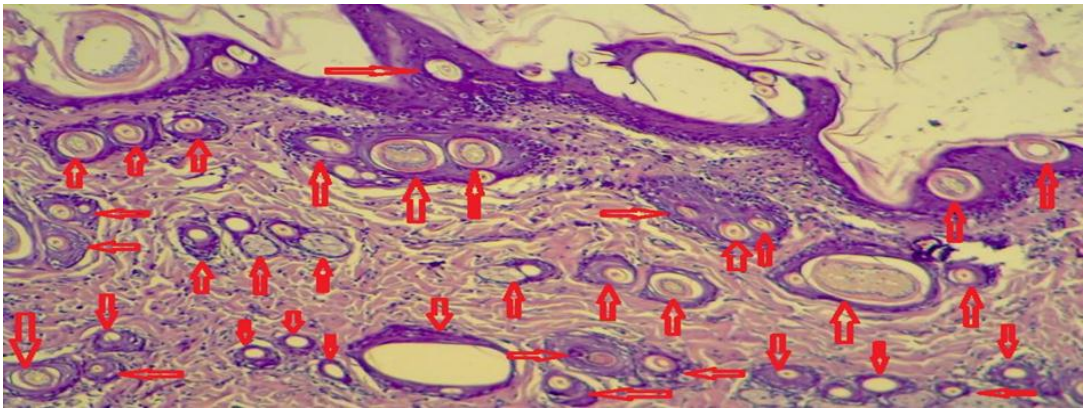

Figure 7. Standard (Minoxidil) group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

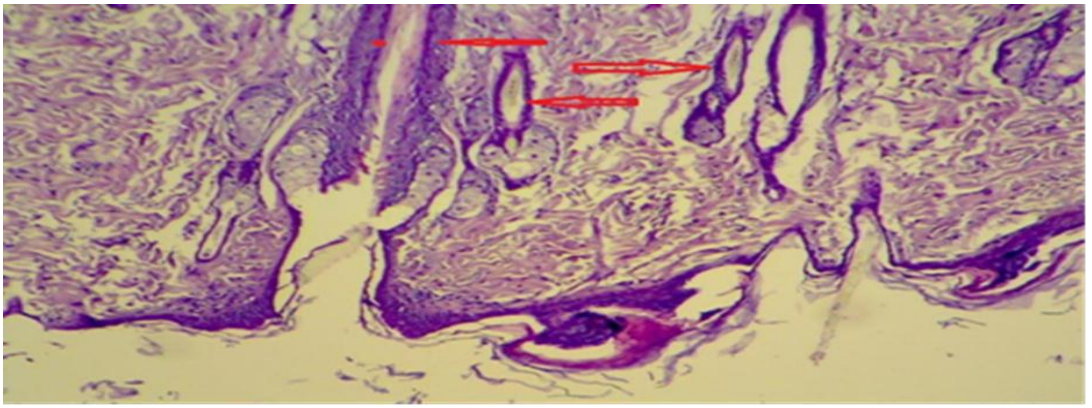

Figure 8. Vehicle group using H&E stain under microscope at 10X power, hair follicles marked with red arrows

Discussion
Effect of PTX on hair weight

R. Rückert *et al.*(2000)work by giving TNF-α and INF-γ and monitor their effects on HF keratinocytes which lead to apoptosis that resembled the CYP administration which produce rise in these cytokines that had a destructive effect on HFs and has induced apoptosis in keratinocytes of the intrafollicular hair bulb and hence decrease hair growth and thickness(27).

The hair weight showed improvement in groups treated with PTX as compared with negative control group. The improvement was stepwise increase with elevating concentration of PTX in our study (table 1). PTX had anti-inflammatory action as O. Rott *et al.* (1993)reported in their study(28). The hair weight measurement revealed a significant increase as PTX 3% group compared with PTX 1%. The influence of enhancing blood flow resulted in improvement of hair growth.

Effect of PTX on follicles number

The results of current study revealed a decline in HFs count in comparison positive control group with negative control group (table 2). These results were consistent with the previous study of T. Ohtani *et al.* (2006) that revealed CYP influence on HFs(29).

As comparing groups that treated with PTX in different concentrations with negative control group showed significant increase in means follicles count (table 2).The number of HFs increased more significant when administered PTX with increasing concentration and showed higher beneficial effect on follicles numbers in PTX 3% group (table 3-5). This increase had been attributed to immune modulation and increased proliferation that reported by study of H. Tilg *et al.* (1993)on PTX pharmacological effect(30). The mean of HF in standard group was significantly increased as comparing negative control group (table 2). Study of J. Han *et al.* (2004)and Messenger *et al.* (2004)reported increase proliferation of HF by minoxidil(31,32).

Effect of PTX on anagen percent

The results of anagen percent revealed significant decrease when compare negative control with control groups (table 3). These findings were in line with previous study of U. Saleem *et al.*(2017), R. Paus *et al.* (2013)and S. Patel *et al.* (2014)that documented CYP influence on HFs and resulted in the transformation from anagen into catagen phase(24,33,34).

The assessment of results revealed there were stepwise elevations with increasing concentration from 1% to 3% in anagen percent in PTX treated groups when compared with negative control group (table 3). PTX have regulating effect on inflammatory cytokines and could improve the follicle cells structures that lead to entrance into anagen phase when proliferation activity restored.

Histological findings

The findings in histopathology of positive control group within current study revealed decrease in HFs and destructive effect of CYP as compared with negative control group (figures 2 and 3).The study of Paus *et al.* (2013) reported that CYP administration induces matrix deterioration. As a result, there was premature catagen formation as well as increase in rate of telogen shedding[35].

The histopathology showed miniaturized hair follicles by inflammatory infiltrate seen in negative control group have been induced to the anagen phase by action of PTX and decreased apoptosis and dystrophic follicle changes in skin areas that affected with CYP. The histological analysis of PTX 3% treated group revealed almost normal hair follicle with regular hair shafts and hair bulb. These results suggested that hair follicle phase could be regulated by PTX after cyclophosphamide-induced damage when compared PTX treated groups with positive control group (figures 3, 4, 5 and 6).

Conclusion

Topical administration of pentoxifylline 3% induced hair growth in cyclophosphamide induced alopecia.

Recommendations

- Increase the concentration of PTX more than 3%
- Testing the effectiveness of PTX in treatment of another type of alopecia such as Alopecia Areata.

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