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Minoxidil nanoliposomes as a hair growth stimulator and a scalp disinfectant

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Abstract: Hair loss (alopecia) continues to be an issue for both sexes. There are multiple ways to reduce the effects of alopecia, one of which is topical minoxidil (MXD). This study aimed to test the effects of minoxidil nanoliposomes (MXD-NLs) on the hair of mice, compared with free MXD and to examine the disinfectant ability of MXD-NLs toward scalp bacteria. To test the study hypothesis, MXD-NLs and free MXD were prepared. Mouse hair was shaved prior to the experiment. MXD-NLs, free MXD, and their vehicles were applied for 15 days. In addition, dermal swabs were used to isolate scalp bacteria and test the inhibitory effect of pretreated media with the two formulations and their vehicles. The results revealed that hair growth in the MXD-NLs -treated group (0.65±0.1cm) was higher than that in the free MXD -treated group (0.53±0.2cm). In addition, MXD-NLs treated media reduced the number of scalp bacteria (p=0.0456) compared with free MXD. These results reveal a novel formulation of MXD with faster hair growth properties and a better disinfectant effect than free MXD. This study can help future researchers to expand and develop MXD-NLs.

Keywords: Hair loss, minoxidil, minoxidil nanoliposomes, hair growth, scalp bacteria.

INTRODUCTION

Hair is a primary characteristic of humans and animals, with substantial physical and social importance. Hair loss or, as stated medically, alopecia is considered a visible factor for any internal changes that happen to a mammal. There are multiple factors that assist in the shedding of hair from the scalp such as inheritability, age, medical conditions, hormonal imbalances, diet and even certain hairstyles (Al Aboud and Zito (2022)). Alopecia occurs with aging, is medically known as androgenic alopecia (Ho *et al.*, 2022).

The human skin, including the scalp surface, provides the body's first line of defense and it is the habitat for a diversity of microorganisms, including bacteria and fungi. Studies have indicated that the scalp microbiome has a relatively low bacterial diversity when compared to other body regions, dominated by bacteria of the genera *Cutibacterium* and *Staphylococcus* (Saxena *et al*., 2021). Microbe and host exchanges on the scalp may play a role in skin homoeostasis and the hair cycle (Polak-Witka *et al.*, 2020). The balance in bacterial population is substantially linked to controlling fungal growth, scalp wellness and diseases (Kerk *et al.*, 2018). The structures of the bacterial communities on the scalp differ from those seen in other parts of the body (Watanabe *et al*., 2020). Scarce data exist on the relationship between the microbial population of the scalp and its impact on hair growth (Pinto *et al.*, 2019).

There are a variety of successful techniques to cure hair loss, these treatments involve either surgery or medication. One such medication is MXD. It is taken without a prescription, in the form of a shampoo or a topical liquid used daily on the scalp to restore hair growth or reduce its loss. Hair transplant surgery is another option for treating permanent hair loss and it is one of the most common treatments. Hair transplantation or restoration surgery can be implemented to get the most out of hair that has fallen out (reviewed in (Llamas-Molina *et al.*, 2022)).

Various formulations using nanotechnology have been used for medical, nutritional and cosmetic purposes. MXD was first known to be a drug able to slow the resistance of blood flow. However, with years of research and experiments, it was discovered as a hair growth stimulator (Eisavi *et al.*, 2019). MXD is abundantly used for androgenic alopecia treatment as a potassium channel opener, allowing more blood flow directly to the hair follicle. With the improvement of nanotechnology in drug formulations, the hair follicle can act as a drug reservoir, which can be beneficial for nano-based MXD (reviewed in (Santos *et al*., 2020)).

Lipids are widely used in the preparation of nano-formulations as in solid lipid nanoparticles, nanostructured lipid carriers and nanoliposomes. They were implemented in biopharmaceutical research to treat skin problems by producing diverse nanoparticle morphologies. Because they are made up of lipids that are similar to those found on the skin, their constituents have been found to be biocompatible, reducing the risk of toxicity and irritation when applied to the skin. Using lipid formulations requires less dose of the drug to achieve its therapeutic effect and reduces the risk of side-effects because the lipids can enhance drug permeation. Several studies showed positive effects of lipids-

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contained nano-formulations in the delivery of MXD (reviewed in (Santos *et al*., 2020)).

In a recent study, MXD was supplied in nanoparticle drug formulation and tested on C57BL/6 mice to monitor the efficiency of these nanoparticles in hair follicles. The results showed that, due to the size of MXD nanoparticles (300-90nm), the efficiency and drug absorption were increased, facilitating the delivery of MXD directly into the hair bulbs from the follicles (reviewed in (Ahmadi-Ashtiani *et al.*, 2020)). An experiment evaluating the toxicity of MXD nanoparticles showed that there was no accumulation of nanoparticles observed over 2 weeks, as well as no irritation observed on the skin of mice (Nagai *et al*., 2019).

This study aimed to test the ability of MXD in NLs as a hair growth stimulant and disinfectant in comparison to free MXD.

MATERIALS AND METHODS

Materials

Chemicals and equipment

Dimethyl dioctadecyl ammonium bromide (DDA) (product code 120150-0050, 50 g) was purchased from DC Fine Chemicals (Spain, SLU Cobalt). MXD powder (patch No: 2022-01-28A, 50g) was purchased from the Greenway Biotech website (Suzhou, China). Cholesterol (product code 117541-0100, 100g) was purchased from DC Fine Chemicals (Spain, SLU Cobalt). An analytical balance (model ADL 500L), hotplate stirrer machine, ultrasonicator, biosafety cabinet, centrifuge, spectrophotometer, and pipette (volume: 1mL and 50mL) were used for the experiment. Other reagents used in this study were methanol, chloroform, saline and distilled water.

Experimental animals

Ten Swiss Albino male mice (weighted 18-25g) were randomly divided into three groups; the two groups where the treatment was applied contained four mice, while the control group contained two mice. The study was approved by the Biomedical Ethics Committee of King Abdulaziz University, Faculty of Medicine (Reference No: 130-22).

Method

Preparation of MXD-NLs suspension

To prepare MXD-NLs, three individual solutions were prepared. A solution of 50mg of DDA was dissolved in 50mL of chloroform and left on a hotplate stirrer until completely mixed. A solution of 50mg of cholesterol was dissolved in 50mL of chloroform and left on a hotplate stirrer until completely mixed. A solution of 50mg of MXD was dissolved in 20mL of methanol (National Centre for Biotechnology Information, 2022) and 30mL of water and left on a hotplate stirrer until completely mixed.

The three solutions were mixed in a ratio of 2:1.5:0.5 (DDA, MXD, cholesterol) and left on a hotplate stirrer overnight until dried and precipitated. Then, the mixture was rehydrated with distilled water and left on a stirrer again until dried. Finally, it was rehydrated with distilled water. The mixture was put through an ultrasonicator for 10 min to agitate the particles and then centrifuged (9500rpm, 90 min, 10°C, five times). Then, the mixture was diluted with normal saline to 4%.

Nanoliposomes vehicle (NLs V) was prepared with the same procedure of MXD-NLs, but without adding MXD.

Preparation of MXD solution and its vehicle

In a beaker, 50mg of MXD was dissolved in 20mL of methanol, and then water was added to obtain 50mL of solvent. When preparing the MXD vehicle (MXD V), the same procedure was repeated without adding MXD. Then, the two mixtures were diluted with normal saline to 4%.

Measurement of particle size and zeta potential

MXD-NLs and vehicle formulations were diluted with normal saline to contain 4% MXD per 1 mL. To analyze the particle size, polydispersity index (PDI), and zeta potential of the MXD-NLs and vehicle, the Zetasizer and DTS1060C zeta cell (Malvern, UK) were used. The scattering angle of the instrument was adjusted to 173°, and the laser wavelength was fixed to 633 nm.

Isolation of scalp bacteria

Four male mice were used for the isolation. A swab was carefully rubbed across selected shaved spots in the dorsal area of each mouse. The bacteria in the swabs were then mixed with 500µL of saline. Next, 125µL of the saline–bacteria mixture was mixed with four different liquid NA media in separate plates. The plates were incubated for 24h at 37°C.

Isolation of scalp bacteria in treated media

First, 125µL of the saline-bacteria mixture was mixed with different nutrient agar (NA) media before hardening. The media were premixed separately with 1mL of 4% MXD-NLs, NLs V, 4% MXD, or MXD V, while the control group was mixed with 1mL of normal saline. After incubating the cultures at 37°C for 24h, the number of bacterial colonies was counted.

Experimental Design

The dorsal area in each mouse was shaved in two halves, 1 day before adding the treatment. Group 1: In the left shaved area, 1mL of MXD-NLs were applied, while 1mL of NLs V was applied to the right shaved area. Group 2: In the left shaved area, 1mL of MXD solution was applied, while 1mL of MXD V was applied to the right

shaved area. Group 3 (negative control): In the left shaved area, 1mL of saline was applied, while 1mL of saline was applied to the right shaved area, to monitor the natural hair growth (Nagai *et al.*, 2019).

The solutions were added once daily at 10:00 a.m. for 15 days. Each day, hair growth was recorded using an iPhone camera at 9:00 a.m. The hair growth was measured on days 1, 15, and 21. In addition, the side-effects of MXD were monitored.

Ethical approval

The study was approved by the Biomedical Ethics Committee of King Abdulaziz University, Faculty of Medicine (Reference No. 130-22).

STATISTICAL ANALYSIS

The analysis of data was conducted using the GraphPad Prism program (ninth edition). The data were conveyed as the mean ± standard error of the mean (SEM). The data in each group were analyzed by one-way ANOVA, while Tukey's multiple comparisons were used to compare two groups. The differences were considered statistically significant when P values were lower than 0.05.

RESULTS

Average size, PDI, and zeta potential

The estimations of average particle size, PDI, and zeta potential for the MXD-NLs and vehicle are presented in table 1.

Table 1: Average size, PDI and zeta potential of the MXD-NLs formulation and vehicle.

Drug	Average size (nm)	PDI	Zeta potential (mV)
MXD-NLs	739.45±15.45	0.5835±0.0655	32.1±1.04
NLs V	86.9±10.115	0.1605±0.0415	-7.595±0.485

Values are expressed as mean ± standard error of mean; MXD-NLs, minoxidil nanoliposomes; NLs V, minoxidil nanoliposomes vehicle; PDI, polydispersity index.

The average size of MXD-NLs was larger than that of NLs V ($p<0.0001$). However, the MXD-NLs formulation was less homogeneous (PDI=0.5835±0.0655) than the vehicle (PDI=0.1605±0.0415). In addition, the zeta potential of MXD-NLs was highly positive (+32.1±1.04 mV), while it was negative in NLs V (-7.595±0.485mV); both differences were significant ($p=0.0055$).

Hair growth monitoring

There were differences in hair growth across the three groups (MXD-NLs, MXD solution and control) through days 1, 5, 8, and 15 (fig. 1). The left side of each mouse was where the treatment was added, whilst the right side was where the vehicle was added.

Images of Swiss Albino male mice were taken daily at 9:00 a.m. Mice were treated once daily for 15 days with MXD-NLs, MXD solution, or saline at 10:00 a.m. Hair was shaved a day before applying the treatment. In the left shaved area, the treatment was applied, while the vehicle was applied to the right shaved area. The efficacy of MXD-NLs on hair growth was remarkably higher than that of MXD solution and saline. MXD-NLs, minoxidil nanoliposomes; MXD, minoxidil.

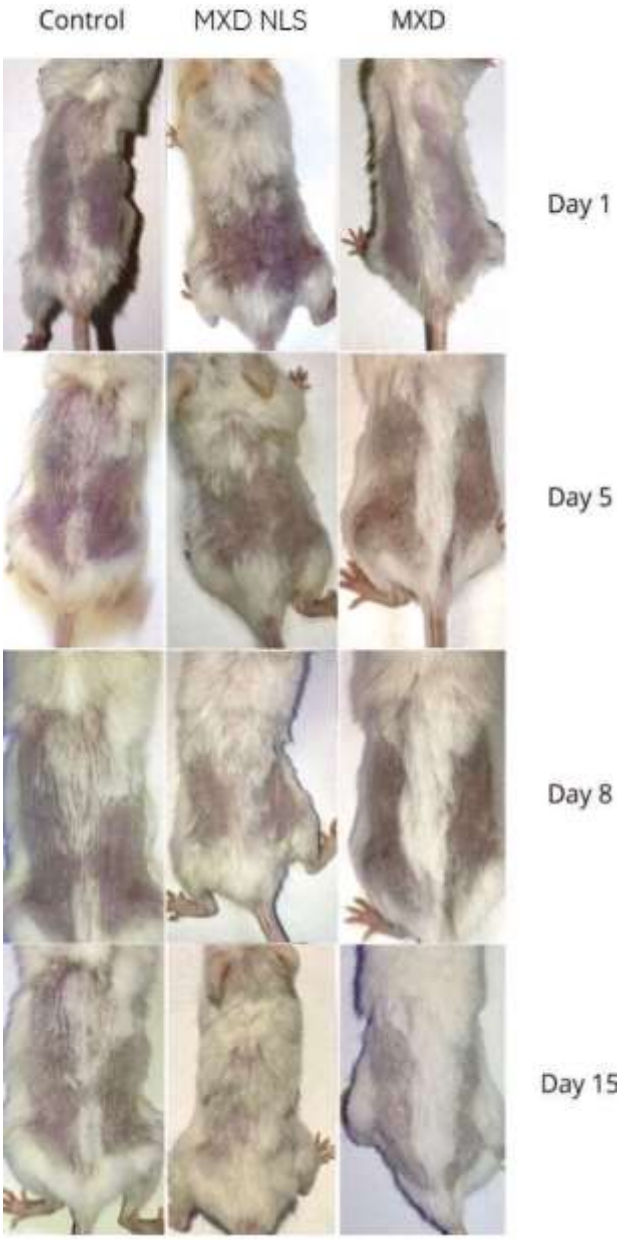

Fig. 1: The difference in hair growth across the control, MXD-NLs and MXD groups (n = 10).

A greater increase in hair growth was observed in the MXD-NLs group compared to the MXD and control groups. Unexpectedly, both sides of the MXD-NLs group showed the same rate of hair growth.

Within 1 week of applying the treatment, MXD and MXD-NLs groups both showed redness around their faces (Fig. 2 and 3). However, the redness and side-effects for the MXD group faded after 5 days. Meanwhile, in the MXD-NLs group, the side-effects faded after 1.5 weeks. Fortunately, no skin rashes were observed on the application area.

Fig. 2: The side effects of applying MXD in the MXD solution group.

Facial redness was the most visible side-effect within the MXD-treated group. The picture on the left was taken 1 week after the application of MXD solution, which was when the side-effects started to appear. The picture on the right was taken when the redness disappeared 5 days after the side-effects appeared. MXD, minoxidil.

Fig. 3: The side effects of applying MXD in the MXD-NLs group.

Facial redness was the most visible side-effect within the MXD-NLs-treated group. The picture on the left was taken 1 week after the application of MXD-NLs, which was when the side-effects started to appear. The picture on the right was taken when the redness disappeared, 1.5 weeks after the side-effects appeared. MXD-NLs, minoxidil nanoliposomes.

Hair measurements

On day 15, the mouse hair was measured (fig. 4).

The control group was only treated with saline. The efficacy of the MXD-NLs was drastically greater than that of the MXD solution group (p=0.9901) at a confidence interval of 95%. MXD, minoxidil; MXD V, minoxidil vehicle; MXD-NLs, minoxidil nanoliposomes; NLs V, minoxidil nanoliposomes vehicle.

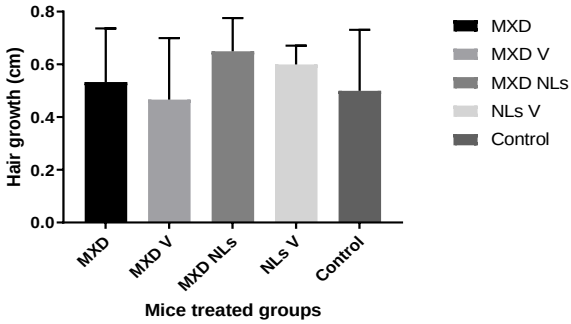

Fig. 4: The average hair length in the treated group (n=10).

The mean value of hair growth in the MXD-NLs-treated group was 0.65±0.1cm. This was higher than all other groups, although the differences were not significant (MXD, p=0.9901; MXD V, p=0.9492; NLs V, p=0.9995; control, p=0.9670).

The mean value in the control group was 0.5±0.2cm, which was surprisingly higher than the value of MXD V, although the difference was not significant (p>0.9999).

On day 21, the hair length was measured (fig. 5).

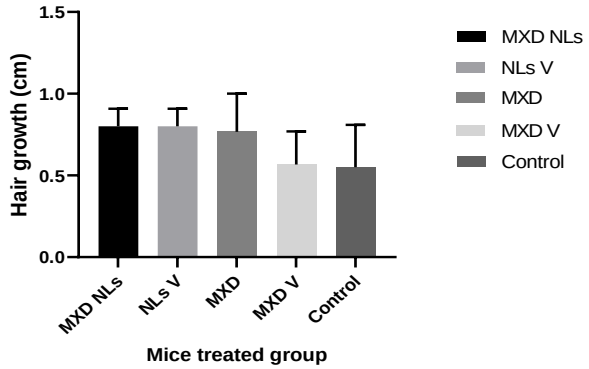

Fig. 5: The average hair length in treated groups on day 21 (n=10).

The control group was only treated with saline. The efficacy of MXD-NLs was drastically greater than that of the control group (p=0.8559) at a confidence interval of 95%. MXD, minoxidil; MXD V, minoxidil vehicle; MXD-NLs, minoxidil nanoliposomes; NLs V, minoxidil nanoliposomes vehicle.

The mean value of hair growth in the MXD-NLs-treated group was 0.8±0.1cm. This was the same as the NLs V group and higher than the other groups. The mean values of hair growth in the MXD V group and the control group were nearly the same. However, the differences between

the MXD-NLs group and the other groups were not significant (MXD, p=0.9999; MXD V, p=0.9084; NLs V, p=0.9999; control group, p=0.8559).

The mean value of hair growth of the control group was 0.55±0.5 cm, which was close to the mean value of hair growth in the MXD group (0.56±0.3 cm); however, the difference between them was not significant (p>0.9999).

Isolation of scalp bacteria in treated media

Dermal swabs were taken from the shaved scalps to isolate the bacterial flora. These swabs were diluted in 1 mL of normal saline and added to different NA media, treated with MXD-NLs, NLs V, MXD, MXD V, or normal saline. The number of bacterial colonies was recorded (fig. 6).

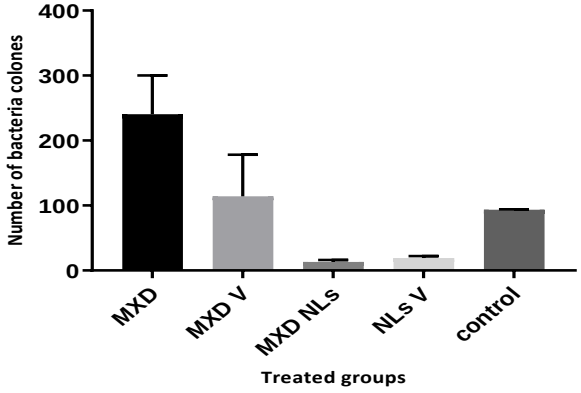

Fig. 6: The number of bacterial colonies in treated media.

The isolated bacteria were manually counted. The control group was only treated with normal saline. The effect of MXD-NLs was significantly lower than that of the control group (p=0.0014) at a 95% confidence interval. MXD, minoxidil; MXD-NLs, minoxidil nanoliposomes; MXD V, minoxidil vehicle; NLs V, minoxidil nanoliposome Vehicle.

The growth of scalp bacteria was affected by different treatments. The number of bacterial colonies in the MXD-NLs group was 13±3 colonies, which was less than that in the MXD-treated group (p=0.0078) and the control group (p=0.0014). However, there were no significant differences between the MXD-NLs and NLs V (p=0.2929).

The number of bacterial colonies in MXD was 241±59.5 colonies, and there were no significant differences between MXD and the control group (p=0.1934), MXD V (p=0.2843), or NLs V (p=0.0456).

The number of bacterial colonies in the control group was 94±0.5 colonies, there were no significant differences between the control group and MXD (p=0.1934), MXD V (p=0.9947), MXD-NLs (p=0.6252), and NLs V (p=0.6801).

DISCUSSION

In this study, the effects of free MXD, MXD-NLs, and their vehicles were tested and compared with normal growth in the saline-treated group.

Considering the advances in nanotechnology, nanoliposomes were chosen for this experiment to facilitate the penetration of MXD through the cell membrane, which is composed of a phospholipid bilayer (reviewed in (Santos *et al*., 2020). The free particles, i.e., those not contained in the nanoliposome, were not removed from the MXD-NLs formulation. This explains the large particle size (739.45±15.45nm) and the low homogeneity (PDI=0.5835±0.0655) of the MXD-NLs formulation compared to the NLs V. However, the MXD-NLs formulation was more stable than the NLs V, as evidenced by the zeta potentials of 32.1±1.04mV and -7.595±0.485mV, respectively.

After the application of the topical treatment, hair growth was monitored. From day 5, mice's hair growth in the MXD-NLs-treated group was much higher than in the free MXD-treated group. The fast hair growth rate in the MXD-NLs-treated group remained until day 15, whilst hair in the MXD-treated group grew slowly in comparison. Several researchers have agreed that the absorption rate of MXD is aided by delivering MXD in nanoparticles, which accelerates the rate of hair growth (reviewed in (Santos *et al*., 2020); Nagai *et al.*, 2019**(**. In addition, nanoliposomes have been effectively used to improve the penetration of drugs through the skin *in vivo* (reviewed in (Salazar, J *et al.*, 2022)).

The MXD-NLs-treated group also showed unexpected hair growth on the vehicle side. This could have been caused by the occurrence of cholesterol in the treatment formula, which has long been considered to have a positive influence on hair growth. In addition, studies have suggested that cholesterol could play a major role in hair shaft formation (Palmer *et al*., 2019)

The mice at the beginning of the second week appeared to show side-effects of the treatment, with a certain redness around the eyes and nose. Both the MXD and the MXD-NLs groups had the same side-effects. The redness could have been due to the sudden strong flow of blood, as MXD can be used to open certain potassium channels in the cells to increase blood flow (Knutsen *et al.*, 2018). The difference in the duration of recovery from side-effects of MXD (1 week) and MXD-NLs (1.5 week) could have been due to the strength of absorption in the MXD-NLs-treated group. NLs enable better absorption and cell penetration due to their smaller particles. The faster absorption could also mean a slower release from the cell (reviewed in (Du and Yin, 2022)).

Scalp bacteria were isolated in different NA media mixed with the various treatment formulations. The results showed that the MXD-NLs (p=0.6252) and NLs V (p=0.6801) groups had less growth than the control group.

Therefore, nanoliposomes had an inhibitory effect on the growth of scalp bacteria. According to a previous study, *Staphylococcus* is the most common bacteria on the skin and mucosal membrane of humans and other mammals. It is an opportunistic pathogen that can also be virulent once inside the body (reviewed in (Fanaei Pirlar *et al.*, 2022)). According to our results, nanoliposomes had a disinfectant effect on the growth of scalp bacteria, which can play a beneficial role in the health of the scalp.

The outcomes of this experiment confirm that the usage of nanoliposomes accelerated the rate of hair growth. Compared to the MXD group, MXD-NLs yielded better results. Although side-effects in the MXD-NLs group were visible for a longer time than in the MXD group, they faded with time.

CONCLUSION

This study yielded positive results regarding the effects of MXD-NLs on hair growth, which can help in finding new ways to increase hair growth, whether it be to cure alopecia or increase hair growth at a faster rate. Although side-effects were visible, future experiments can investigate their prevention. In addition, the disinfectant effect of MXD-NLs maintained the stability of the scalp flora and prevented the overgrowth of opportunistic bacteria. As the usage of MXD in medicine continues to expand, MXD-NLs will find new applications. This study can aid future researchers in finding a solution for hair loss and in preventing the side-effects of MXD-NLs.

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