Nicotinamide Mononucleotide Application in Cosmetics Products with Potential Excellent Efficacy

Yan Long[1], Aixuan Lin[1], Chengkang Tan[1], Chunmei Peng[1], Shentao Guo[2], Siqi Mo[2], Shiwei Li[2], Ruixue Liu[1], Qunying Leng[1]
Guangdong Bawei Biotechnology Corporation, Guangzhou, China[1]
Guangdong Youzhi Testing Technology Corporation, Guangzhou, China[2]
5th, Xinbei Road, Baiyun District, Guangzhou, China

*Yan Long, 13570939832, 2881860771@qq.com

Abstract

Nicotinamide mononucleotide (NMN) is an important precursor for the synthesis of nicotinamide adenine dinucleotide (NAD+) in the human body, which can be converted into NAD+ to exert various physiological functions. At present, the application research of NMN is mainly focused on medical drugs and health food, while the application in cosmetics products is nearly blank. In this paper, the safety of NMN was verified by the Chick Chorioallantoic Membrane (CAM) test and Patch test, the results show that there was no irritation in both tests. The stability of NMN was tested, and the degradation rate of NMN placed at 45℃ for 2 weeks was close to 72.67%, in the simple basic skin care formula, the degradation rate at 45℃ for 1 month was close to 95.7%, it was relatively stable under other conditions. In vitro experiments, which including inflammatory factor NO inhibition test, and promoting Pro-collagen synthesis test, and in vivo experiment verified that NMN has a certain soothing, firming and anti-wrinkle efficacy. When the concentration of NMN was 0.003%, the inhibition rate of inflammatory factor NO was 16.86%. When the concentration of NMN was increased to 0.005%, the type I collagen increase rate was 45.19%. Additionally, in the volunteer use test, the skin rebound time decreased by 18.14% after using the sample for 28 days. In this study, the efficacy of NMN applied in cosmetics was confirmed, indicating that it has great value and application prospects as a functional ingredient of cosmetics.

Keywords: Nicotinamide mononucleotide, stability, cosmetics application, in vitro test, efficacy test

1. Introduction

With the improvement of living standards, people pay more and more attention to beauty,

therefore anti-aging has become an eternal topic. According to research, the content of nicotinamide adenine dinucleotide (NAD+) in the body gradually decreases in the process of aging[1], and NAD+ is an important coenzyme in cellular redox reactions. In humans, abnormalities in the process of NAD+ metabolism will cause the production of various aging-related diseases. NAD+ plays an important role in various biological processes such as cell death, aging, gene expression, neuroinflammation, and DNA repair[2]. When the NAD+ level in the body is restored, the disease status can be improved and even prolong lifespan. Therefore, NAD+ has now become an important target for anti-aging and related diseases. Nicotinamide mononucleotide (NMN) is an intermediate product of NAD+ biosynthesis, one of the key precursors of NAD+[3], and the most direct route to NAD+ synthesis[4]. NMN can be converted into NAD+ to play various physiological functions in the human body.

In terms of application, the research of NMN mainly focuses on the field of medical drugs and health food. For example, in the field of medical drugs, it could alleviate and improve ischemic brain injury, such as Alzheimer's disease (AD)[2,5], Parkinson's (PD)[6], and strengthen the barriers to angiogenesis[7], etc.. In the field of health food, studies shown that oral administration of NMN to mice could effectively reduce the age-related physiological decline in mice, also shown that NMN did not have any apparent toxic or harmful effects. When lower concentrations of NMN were taken orally, NMN could be rapidly absorbed within 30 minutes, effectively transported to the blood circulation, and immediately converted into NAD+ in the main metabolic tissues[8]. While these related experiments were tested more in animals than in human beings. At present, as a cosmetic raw material, NMN has already passed the new raw material audit in China. However, little work has been conducted on NMN in the field of cosmetic applications. In patent WO 2016145912 A1[9], NMN was applied in cosmetics, in vivo experiments at different ages have been carried out, and the wrinkles and sagging of the skin have been improved, but no research on related safety, stability, and vitro experiments has been carried out.

This research will focus on the application of NMN in cosmetics, investigate the safety of NMN in cosmetics, and explore the stability of NMN and application formulations containing NMN ingredient, further study the efficacy of NMN by in vivo and in vitro emperiments. In short, we try to explore the feasibility and efficacy of NMN in the field of cosmetics.

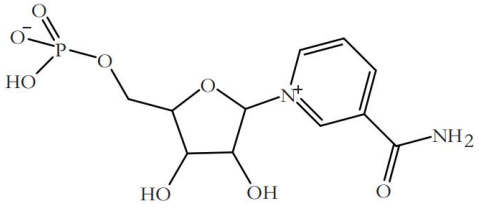


Figure 1 the structure of the nicotinamide mononucleotide (NMN).

2. Materials and Methods

2.1 Safety Test

The safety of the samples was tested by Chick Chorioallantoic Membrane (CAM) test and Patch test.

2.2 Stability Test

The stability of 0.15% NMN aqueous solution and simple basic skin care formula containing NMN (addition level of 0.5% ~ 10%) was evaluated after a period of time at 45℃, sunlight, 5℃, room temperature, high-low temperature cycle, and -15℃. The content of NMN was analyzed by the High-performance liquid chromatography (HPLC) (Palo Alto, CA, USA) method.

HPLC conditions for analysis as follows: The analysis was performed on an Agilent 1260 series system, chromatographic column model: C18 250mm*4.6mm*5um, column temperature 30℃, Mobile phase A: weighed 13.6 g of KH_2PO_4, and 2.7 g tetrabutylammonium hydrogen sulfate were dissolved in 800 mL first-grade water, adjusted to pH 3.5 ± 0.05 with KOH solution, replenished with water to 1000 mL, added with 64 mL of methanol, stirred evenly, finally filtered with a 0.45 um micro porous membrane and degassed by ultrasonic. Mobile phase B: measured 700 mL of methanol with a graduated cylinder and diluted to 1000 mL with first-grade water, and used it after ultrasonic treatment. The gradient conditions as follows: 0% B, 0-12, 21-30 minutes, 6% B, 12-17 minutes, 70% B, 17-21 minutes, the mobile phase flow rate was 0.8 $mL \cdot min^{-1}$, and the injection volume was 10 μL. Monitor the UV spectrum at 254 nm.

2.3 In vitro soothing efficacy test

Cytotoxicity test: the concentration of RAW264.7 cell suspension (China Type Culture Collection Center, Wuhan, China) was adjusted, and added to a 96-well cell plate, cultured overnight at 37℃ in 5% CO_2 for the test. The samples were diluted with cell culture medium to different concentrations and added to the cell plate respectively, and cultured at 37℃ in 5% CO_2 for 20-48 hours. The culture medium was discarded and washed cells 1-2 times with PBS, and cells added MTT (Sigma, St.Louis, MO, USA) solution to continue the culture, the medium was discarded after the culture and added DMSO (Aladdin, Shanghai, China), then shaken after mixing. The OD value (Optical Density) was measured at 570 nm, and the sample concentration without obvious cytotoxicity was screened for the next experiment according to the cell morphology.

NO release test: the concentration of RAW264.7 cell suspension was adjusted, and added to a 96-well cell plate, cultured overnight at 37℃ in 5% CO_2 for detection. The test samples were diluted with LPS (Lipopolysaccharide) containing cell culture medium (Sigma, St.Louis, MO, USA) to a non-cytotoxic concentration and added to the sample. The LPS-containing medium was used as a positive control, and the LPS-free cell culture medium was used as a blank control. The

cells were cultured for 20-24 hours after adding the sample. After that, 50 μL of the culture medium was put into a new cell plate, and an equal volume of Griess reagent (Sigma, St. Louis, MO, USA) was added and mixed well. After 10 min reaction in the dark, the OD value was detected at 540 nm.

2.4 In vitro anti-wrinkle and firming efficacy test

Cytotoxicity test: the concentration of HFF-1 cell suspension (Chinese Academy of Sciences, Shanghai, China) was adjusted, and added it to a 96-well plate, cultured at 37°C in 5% CO_2 for 24 hours. Prepared a series concentrations of gradient samples, and added 100 μL/well respectively in a 96-well plate, and incubated for 24 hours at 37°C in 5% CO_2. Then the culture medium was discarded. Cells were washed twice with PBS, added 50 μL/well of MTT solution, and incubated for 2-6 hours at 37°C in 5% CO_2. The medium containing MTT was discarded and added DMSO with 150μL/well, shaked for 5-10 minutes, the OD value was measured at 570nm. Select the concentration with no obvious cytotoxicity for protein content determination experiments.

Determination of type I collagen content: the concentration of HFF-1 cell suspension was adjusted, and added to a 96-well plate, cultured at 37°C in 5% CO_2 for 24 hours. The previous medium was discarded. Then induced modeling with UVB, and added 100μL of DMEM medium (Gibco, Carlsbad, CA, USA) containing samples to each well after irradiation. To set up a blank medium control group and a culture medium containing TGF-β (Sigma, St.Louis, MO, USA) was the positive control group, which was cultured at 37°C in 5% CO_2 for 24±2 hours. After the incubation, the content of type I collagen was identified according to the instructions of the ELISA kit (Abcam, Cambridge, UK).

Determination of elastin content: the concentration of HFF-1 cell suspension was adjusted, and added it to a 96-well plate, cultured at 37°C in 5% CO_2 for 24 hours. The previous medium was discarded. Added 100 μL DMEM medium containing sample to each well, and set the medium blank control group and the medium containing TGF-β as the positive control group. The sample cultured at 37°C in 5% CO_2 for 24± 2 hours. After the incubation, the elastin content was needed to be measured according to the instructions of the ELISA kit.

2.5 In vivo efficacy test

34 healthy Chinese volunteers, with an average age of 39.3, were selected for a single-blind efficacy test, which including anti-wrinkle and moisturizing efficacy tests. The sample containing 1% NMN and the negative sample(base without NMN) were used by volunteers twice a day and lasted for 28 days. To take an appropriate amount of sample on each face respectively and gently massaged until completely absorbed. The indicators of wrinkle parameters, moisture, and skin

rebound time were assessed on volunteers before, and after 14, 21, and 28 days by two instrumental methods, Antera 3D (Mirvex, Dublin, Irelan) and DermaLab® Combo (Cortex, Copenhagen, Denmark).

2.6 Statistical analysis

In vivo efficacy test, the SPSS (SPSS Inc., Chicago, IL, USA) was used as statistical analysis software and Shapiro-Wilktest was used to assess the significance of the datas. If Sig. (two-sided) > 0.01, the distribution was normal, and a paired t-test was carried out. Sig. (bilateral) < 0.01, If the distribution was not normal, the Wilcoxon test was performed. In vitro tests, GraphPad Prism (GraphPad software, La Jolla, CA, USA) was used as statistical analysis software, and the statistically significant differences were: (n.s) $p > 0.05$, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, and (****) $p < 0.0001$.

3. Results

3.1 Safety test

The results of the CAM test were given in Table 1. Whether it was an aqueous solution of 1% NMN or a simple basic skin care formula containing 1% NMN, the results were non-irritating. Among the 31 volunteers in the patch test, the results were all negative, indicating that NMN was safety enough to used in cosmetics products.

Table 1 CAM test results. Six parallel experiments were performed for each sample. ES was the mathematical sum of the score of 6 chicken embryos, ES≤12: not/light eye irritation; 12＜ES＜16: medium eye irritation; ES≥16: strong eye irritation

Sample	Test Results
The aqueous solution of 1% NMN	Not eye irritation（ES=1）
The simple basic skin care formula containing 1% NMN	Not eye irritation（ES=4）

3.2 Stability test

According to the results of stability tests in Table 2, it could be seen that NMN had poor stability at high temperature. After being placed at 45℃ for two weeks, the degradation rate was 72.67%. Similarly, it could also be observed that the simple basic skin care formula containing 1% NMN had poor stability at high temperature, and the degradation rate was 95.7% at 45℃ for 1 month, and the degradation rate at high-low temperature cycle was 41.6%. It was relatively stable under other test conditions such as -15℃, sunlight, and room temperature. Indicating that NMN has poor stability at high temperatur, and the high temperature stability deteriorates with time.

Table 2 The results of stability test. The measurements of NMN were tested at 45℃, sunlight, 5℃, room temperature, high-low temperature cycle, and -15℃. The 0.15% NMN aqueous solution was placed for two weeks, and the simple basic skin care formula containing 1% NMN was stayed for one month under these conditions respectively.

Sample	Test condition	Residue(%)	Residual rate(%)	Degradation rate(%)
The aqueous solution of 0.15% NMN	-15℃	0.15	100	0
	45℃	0.041	27.33	72.67
	High-low temperature cycle	0.11	73.33	26.67
	Sunlight	0.15	100	0
	5℃	0.15	100	0
	Room temperature	0.15	100	0
The basic skin care formula containing 1% NMN	-15℃	0.838	83.8	16.20
	45℃	0.043	4.3	95.70
	High-low temperature cycle	0.584	58.4	41.60
	Sunlight	0.807	80.7	19.30
	5℃	0.881	88.1	11.90
	Room temperature	0.743	74.3	25.70

3.3 In vitro tests

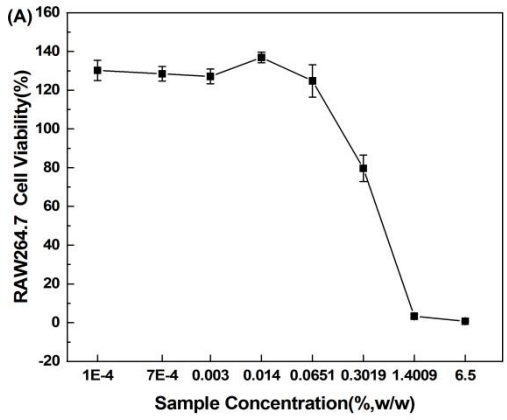
3.3.1 In vitro soothing test

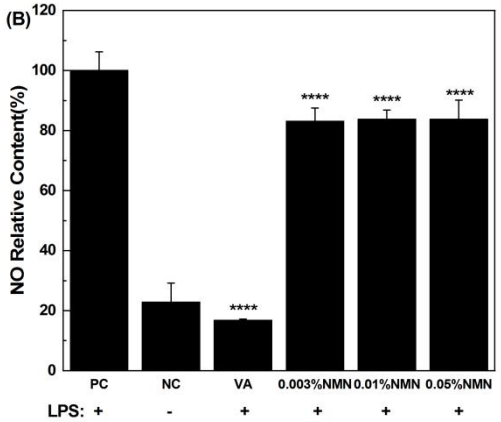
As shown in Figure 2 (A), the RAW264.7 cell viability of the sample at a concentration of 0.065% was 124.78%, and shown that it has no cytotoxicity, so the concentrations of 0.05%, 0.01%, and 0.003% were selected to carry out the experiment. According to the test result in Figure 2 (B), it could be seen that the NC group was compared with the PC group, $P<0.0001$, the results shown an obvious difference between them. The relative content of NO in the NC group was less than or equal to 50%, which shown that this experiment was established. When the

sample group compared with the PC group, P<0.0001, the results shown extremely significant differences, and NO inhibition rates of the sample group were 16.86%, 16.17%, and 16.17% at the concentrations of 0.003%, 0.01%, and 0.05% ,respectively (Figure 2 (C)). Compared with the NC group, there was a significant difference, which indicating that NMN had a good soothing efficacy.

3.3.2 In vitro anti-wrinkle test

HFF-1 cytotoxicity results were shown in Figure 2 (D), under the concentration of 0.1001% of the sample, the viability rate of HFF-1 cell was 86.33%, which shown that it has no cytotoxicity. Therefore, the concentration of 0.005%, 0.02%, and 0.1% were selected to measure the content of type I collagen and elastin. According to the experimental results, compared with NC group, the increase rate of type I collagen and elastin in the PC group was higher than or equal to 20%, which shown that this experiment was effective. When concentrations of sample were 0.005%, 0.02%, and 0.1%, the content of type I collagen was 0.26 ± 0.039 ng/mL, 0.23 ± 0.034 ng/mL, and 0.24 ± 0.015 ng/mL (Figure 2 (E)) separately, at the same conditions the content of elastin was 0.39±0.010 ng/mL, 0.39±0.003 ng/mL, and 0.39±0.004 ng/mL (Figure 2 (G)) respectively. As shown in Figure 2 (F, H) compared with NC group, when the sample concentration was 0.0050%, the increase rate of type I collagen was 45.19%, P<0.05; while the content of elastin has not grown. The increase of the Collagen I indicated that NMN has a good anti-wrinkle effect.





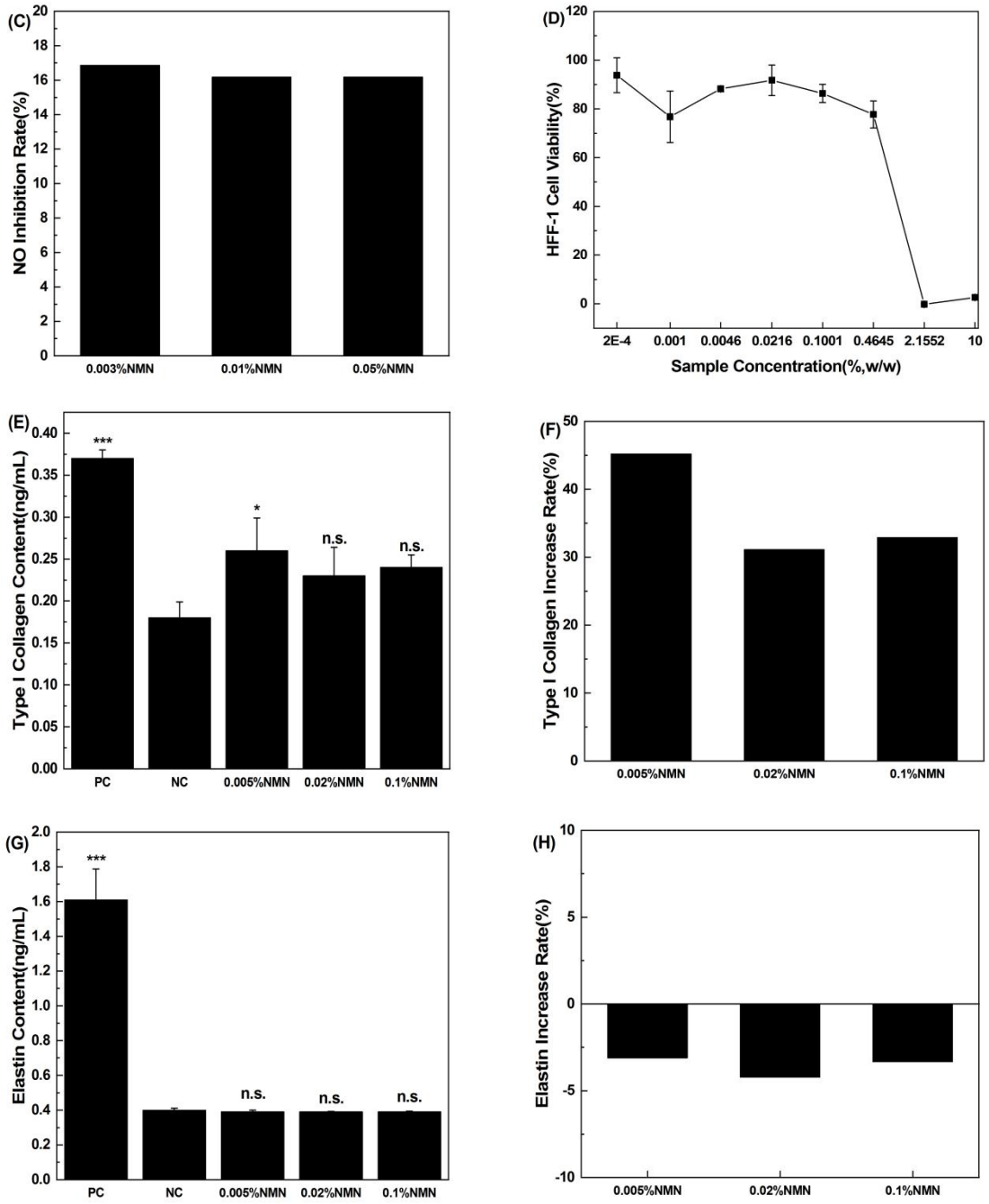
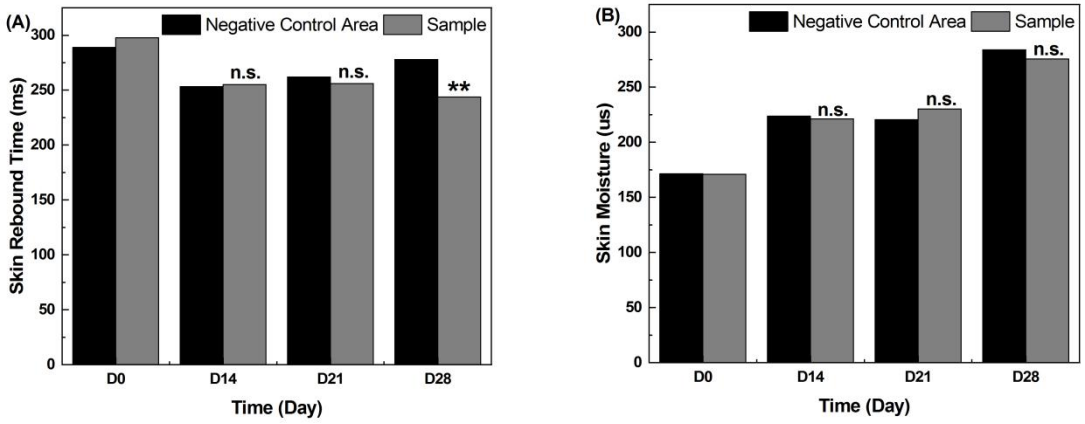


Figure 2 The results of in-vitro cell culture experiments. (A) Toxicity detection of different sample concentrations in RAW264.7 cells. (B) LPS induced inflammatory response in RAW 264.7 cells, the anti-inflammatory activity of NMN aqueous solution was confirmed by measuring the concentration of NO in cell supernatant. In the experiments, PC was the positive control, NC was blank control, VA is the reference sample and NO relative content (%)=$OD_{sample}/OD_{PC}\times100\%$. (C) The results of NO inhibition rate, when $P<0.01$ and NO inhibition rate≥ 10%, the sample could be believed had soothing effect, NO inhibition rate (%)=$(OD_{PC}-OD_{sample})/OD_{PC}\times100\%$. (D) Toxicity detection of different sample concentrations in HFF-1 cells. (E) HFF-1 cells were used as cell model for the study of cosmetic products containing 1% NMN to promote the synthesis of type I collagen. HFF-1 cells were exposured by UVB, then NMN aqueous solution was added followed by UVB treatment, and the content of type I

collagen was measured by ELISA Kit. (F) The results of the increase rate of type I collagen. (G) The content of elastin was measured by ELISA Kit. (H) The results of the increase rate of elastin. (The protein content of the positive control group was increased by greater or equal to 20% compared with the blank control group, which indicated that the test was effective. The contents of collagen and elastin of the sample group were compared with the blank control, $P<0.05$, and when both of them were higher than the blank group, it could be thought that the sample had certain anti-wrinkle and firming effect. Increase rate (%)=(T/C-1)×100%, T—the average value of collagen or elastin in the experimental group, C—the average values of collagen or elastin in blank/solvent group)

3.4 In vivo efficacy test

From Figure 3, we observed that after using the sample containing 1% NMN for 28 days, when compared with the negative control (the same basic formula only without NMN), there was a clear firming efficacy be detected. However, there was no anti-wrinkle or moisturizing effect be observed. After using the sample for 14 days, 21 days, and 28 days, the skin rebound time decreased by 14.3%, 13.95%, and 18.14% ($P<0.01$), respectively. When compared with the negative control, the decrease of skin rebound time has significant difference which shown that NMN had an obvious firming efficacy. The length of wrinkle also decreased during the use period, but compared with the negative control it has no statistically significant, while it had a tendency to improve wrinkles. The improvement of skin moisture was finally increased by 61.21% during the period of the test, however compared with the negative control area, there was no significant difference. Therefore, NMN improved the firmness of volunteer's skin and has a good firming effect.



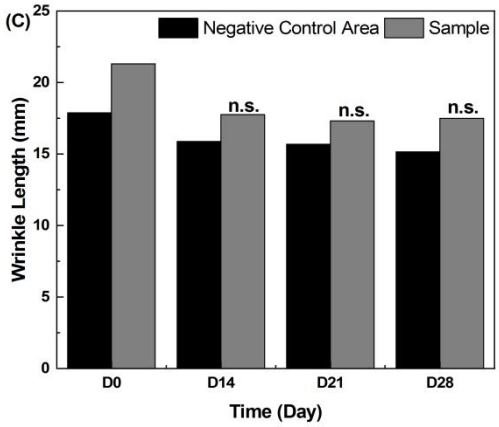


Figure 3 The results of the volunteers' efficacy tests, (A) The results of rebound time of skin before and after using the samples; (B) The results of the length of wrinkle before and after using the samples; (C) The results of moisture of skin before and after using the samples.

4. Discussion

From the perspective of applying the new functional ingredients in the cosmetics products, NMN is one of the potential options. It was confirmed with 2 different methods of safety test, including CAM test and Patch test, and both of them were demonstrated good safety results. In term of efficacy, in vitro cell experiments verified that NMN had good efficacy of soothing and anti-wrinkle. At the same time, the results of in-vivo efficacy tests proved that NMN has a obvious firming effect. Combined with in vivo and in vitro efficacy experiments could support that NMN has good soothing and anti-aging efficacy as a cosmetic raw material.

While due to the poor stability of NMN at high temperature, the degradation rate of NMN solution and the basic skin care formula containing 1% NMN under 45℃ were rather high. In order to solve this problem we currently try to improve the stability of NMN through nano-encapsulation technology. Moreover, the mechanism and pathway of NMN's funtion on skin and the property of transdermal absorption are still unclear, so further researches in this area are needed to solve relavent problems and better understanding of this new potential cosmetic functional ingredient.

5. Conclusion

In this study, in the field of cosmetics application NMN has good soothing, anti-wrinkle, and firming efficacy, and it is also safety enough. At the same time, NMN has good water solubility

and could be applied to various formulation systems. Further efforts are needed to solve the problem of stability under high temperature and transdermal absorption on the skin. Combining the performance of NMN, it is expected to become a compelling raw material in functional cosmetics.

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