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Study on the Efficacy of Recombinant Type III Human Collagen in Skin Care Cosmetics

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Abstract: *The aim of this study was to understand the efficacy of recombinant type III human collagen in skin care cosmetics. A blank control group and a recombinant type III human collagen sample group with concentrations of 0.0025% and 0.01% respectively were set up to understand the skin care efficacy at different concentrations. The moisturising effect test, repair effect test and anti-wrinkle effect test were carried out. The results showed that the recombinant type III human collagen sample at a concentration of 0.0025% produced an effect on skin care; the recombinant type III human collagen sample at a concentration of 0.01% produced a very significant effect on the skin.*

Keywords: *recombinant type III human collagen; moisturizing effect; repair effect; anti-wrinkle effect*

1. Introduction

Collagen, as a structural protein widely existing in organisms, is the main component of skin, cartilage, arterial and vascular walls and other tissues. It has good biological characteristics, mechanical properties, biocompatibility and degradability, and can effectively promote the growth of cells. Collagen is mainly extracted from animal tissues and obtained by high density fermentation of genetically engineered bacteria. Collagen is mainly extracted from animal tissue and produced by high-density fermentation with genetically engineered bacteria. Because most of it is water insoluble protein, molecular breakage may occur when heated, which makes it less processable and poses a risk of biological contamination[1-2]. Collagen is the main protein in human structural tissues, accounting for 30%–40% of the total protein in the human body. It is an essential component of a variety of tissues and organs in the human body, so it is often called "life scaffold". At present, 28 types of collagen have been found in human, and the ones related to human skin are type I collagen and type III collagen. Type III collagen is a slender network structure with the ability to maintain cell repair and regeneration. The proportion of type III collagen determines the elasticity and fineness of human skin. Type I collagen, a coarse fibrous structure, is used to maintain cellular support for human skin[3-4]. Recombinant type III human collagen is a full-length or partial amino acid sequence fragment encoded by a specific type of human collagen gene, or a combination of functional fragments containing human collagen prepared by DNA recombination technology. Recombinant type III human collagen has good water solubility and high biological activity, and its performance is better than that of natural human collagen. It has a good application prospect in biomedical materials, beauty cosmetics, food and health care and other fields[5]. The effect of recombinant type III human collagen concentration on skin care and cosmetics was studied to explore the moisturizing, repairing and anti-wrinkle effects of skin care cosmetics.

2. Materials and methods

2.1. Materials

Table 1: Experimental Sample Information

Name	Concentration	Category	Production date/batch number	Quantity and specification	Physicochemical property	Solubility	Storage condition
Recombinant human type III collagen (CL90)	0.5%	Raw material	July 29, 2022/lot 220729	2 tubes *10 mL/ tube	Colourless liquid	Water soluble	4℃ dark

The test cells used included keratinocytes, fibroblasts and 3D epidermal skin model, all of which were provided by Guangdong Biocell Biotechnology Co., Ltd., and the isolated skin tissue used for the

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test was also obtained by the company. The equipment used included carbon dioxide incubator, clean bench, microscope, and UVA irradiator. The sample information is shown in Table 1.

2.2. Methods

2.2.1. Test method for moisturizing effect

Based on the 3D epidermis skin model, the moisturizing efficacy of the samples to be tested was evaluated by detecting the changes of the water content of the model skin.

2.2.2. Test method for repair effect

Detecting the migration ability of keratinocytes based on keratinocytes; detecting the migration ability of the fibroblasts based on the fibroblasts; the anionic surfactant sodium lauryl sulfate (SLS) was applied to stimulate the 3D epidermis skin model, and detecting the changes of tissue morphology, filament aggregate protein (FLG), loricrin (LOR), transglutaminase1 (TGM1) and hyaluronic acid receptor (CD44) contents of the skin model after the treatment.

2.2.3. Test method for wrinkle resistance

After the fibroblasts were irradiated with UVA, the content change of type I collagen (Collagen I) was detected. Based on the isolated skin tissue, detecting the tissue morphology, collagen fiber and collagen type IV (Collagen IV) protein content of the isolated skin tissue.

3. Result analysis

3.1. Moisturizing effect test results

Table 2: Moisturizing Effect Test Group

Group	Sample name	Dosing concentration	Detection model	Detection Indicator	Test method
Control group	/	/	EpiKutis®	Skin water content	Instrument method
Sample set	Recombinant human type III collagen (CL90)	0.5%			
		2%			

Test groups were performed according to Table 2, the models were transferred to 6-well plates (to which 0.9 mL EpiGrowth medium was added in advance), and the test group numbers were noted on the 6-well plates. The control group did not do any treatment, the sample group was added with 0.5% and 2% sample working solution 1.5 μL, respectively, which were evenly distributed on the surface of the model, and incubated in a CO_2 incubator (37℃, 5% CO_2) for 24h. The samples on the surface of the model were cleaned, and the remaining water on the surface was gently wiped dry with cotton swabs. After that, 24 well plates were prepared according to the number of models and marked accordingly. 0.3 mL of EpiGrowth culture solution was added to each well. The 24-well plate containing the model was placed in the clean bench, the lid of the 24-well plate was opened and left to stand for 30 min before measurement. The bottom of the model was dried, the model was circumferentially removed and placed in the probe position of the tester, the probe was pressed for measurement, each model was measured three times and the average value was taken. The test results are shown in Table 3.

Table 3: Detection Results of Skin Water Content

Group	Skin water content (a.u.)	SD	P-value	Lifting rate
Control group	29.81	0.91	/	/
Recombinant human type III collagen (CL90)0.5%	44.27	1.41	0.000##	48.5%
Recombinant human type III collagen (CL90)2%	87.67	0.54	0.000**	194.1%

As shown in Table 3, compared with the control group, the skin water content of the sample group administered with the concentration of 0.5% significantly increased, that is, when the concentration of recombinant human type III collagen was 0.0025%, it was effective for moisturizing the skin. When the drug concentration was 2%, the skin water content of the sample group increased significantly, with the increase rate as high as 194.1%, which had a very significant effect on skin moisturizing.

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3.2. Repair effect test results

Table 4: Keratinocyte Test Groups

Group	Sample name	Dosing concentration	Detection model	Detection Indicator	Test method
Control group	/	/	Keratinocyte	Cell mobility	Cell scratch
Sample set	Recombinant human type III collagen (CL90)	0.5%			
		2%			

The test groups of keratinocytes are shown in Table 4[1]. Cells were inoculated at an inoculum density of 3.8×10^5 cells/well into 6-well plates and incubated overnight in an incubator (37℃, 5% CO_2). The sample working solution was configured according to the test groups, and when the cell plating rate in the 6-well plate reached about 70%–80%, the drug was administered in groups according to the test groups, with the drug amount per well-being 2 mL, and three wells were set in each group. The culture was continued for 24 h in an incubator (37℃, 5% CO_2). After incubation of the samples, scratches were applied. After the scratching, the cells were washed with PBS three times, and added with normal cell culture medium for culture in an incubator (37℃, 5% CO_2) for 24 h. The test results are shown in table 5.

Table 5: Keratinocyte Migration Result

Group	Average relative mobility	SD	P-value	Lifting rate
Control group	1.00	0.07	/	/
Recombinant human type III collagen (CL90) 0.5%	1.12	0.07	0.011*	12.0%
Recombinant human type III collagen (CL90) 2%	1.43	0.06	0.000**	43.0%

As shown in Table 5, compared with the control group, the relative cell mobility of the sample group was significantly increased when the concentration was 0.5%, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce effects on skin repair. When the dosing concentration was 2%, the relative mobility of sample cells was significantly increased, with the increase rate reaching 43.0%, indicating that it had very significant effect on skin repair.

Table 6: Fibroblast Test Ggroups

Group	Sample name	Dosing concentration	Detection model	Detection Indicator	Test method
Control group	/	/	Fibroblast	Cell mobility	Cell scratch
Sample set	Recombinant human type III collagen (CL90)	0.5%			
		2%			

The test groups of fibroblasts are shown in Table 6[1]. Cells were inoculated at an inoculum density of 2.8×10^5 cells/well into 6-well plates and incubated overnight in an incubator (37℃, 5% CO_2). The sample working solution was configured according to the test groups, and when the cell plating rate in the 6-well plate reached about 70%–80%, the drug was administered in groups according to the test groups, with the drug amount per well being 2 mL, and three wells were set in each group. The culture was continued for 24 h in an incubator (37℃, 5% CO_2). After incubation of the samples, scratches were applied. After the scratching, the cells were washed with PBS three times, and added with normal cell culture medium for culture in an incubator (37℃, 5% CO_2) for 24 h. The test results are shown in Table 7.

Table 7: Fibroblast Migration Result

Group	Average relative mobility	SD	P-value	Lifting rate
Control group	1.00	0.07	/	/
Recombinant human type III collagen (CL90)0.5%	1.13	0.10	0.034*	13.0%
Recombinant human type III collagen (CL90)2%	1.67	0.05	0.000**	67.0%

As shown in Table 7, compared with the control group, the relative fibroblast mobility of the sample group was significantly increased when the concentration was 0.5%, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce effects on skin repair. When the

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dosing concentration was 2%, the relative mobility of fibroblasts in the sample group was significantly increased, with the increase rate as high as 67.0%, which had very significant effect on skin repair.

Table 8: SLS-EpiKutis® Model-based Test Groups

Group	Sample name	Dosing concentration	Detection model	Detection Indicator	Test method
Control group	/	/	Fibroblast	Tissue morphology, FLG, LOR, TGM1, CD44	H&E, Immunofluorescence, Immunohistochemistry
Sample set	Recombinant human type III collagen (CL90)	0.5%			
		2%			

The model test groups are shown in Table 8. After 1 mL of 0.4% SLS solution was aspirated, 1 mL of PBS was added to prepare the 0.2% SLS working solution, and the models were grouped according to the test results in Table 8. The models were transferred to 6-well plates (with 0.9 mL EpiGrowth medium added in advance) and the test group numbers were marked on the 6-well plates. The control group did not receive any treatment. 12.5 μL 0.4% SLS solution and 12.5μL sample working solution at concentrations of 0.0025% and 0.01% were added to the surface of the sample group. The samples were distributed on the surface of the model and incubated in a CO2 incubator (37°C, 5% CO_2) for 24 h. After incubation, the residual test substances on the surface of the model were washed with sterile PBS solution, and the residual liquid in and out of the model was wiped by sterile cotton swabs. The model used for tissue morphology detection was circumferentially removed and fixed with 4% paraformaldehyde for 24 h, and then tested by H&E staining, photographed and observed under a microscope, and the pictures were collected and analyzed. Combined with specific operation steps of immunofluorescence and immunohistochemistry to detect. The results of tissue morphology are shown in Table 9, FLG immunofluorescence analysis in Table 10, LOR immunofluorescence in Table 11, TGM1 immunofluorescence in Table 12 and CD44 immunohistochemistry in Table 13.

Table 9: Organizational Morphology Results

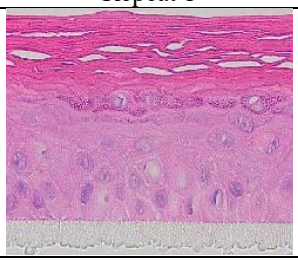
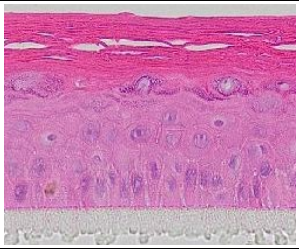
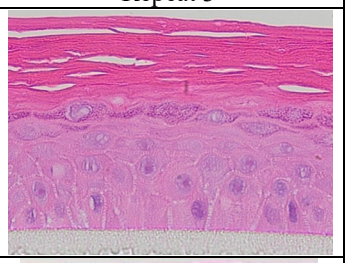
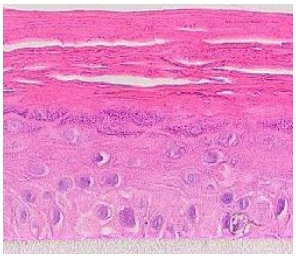
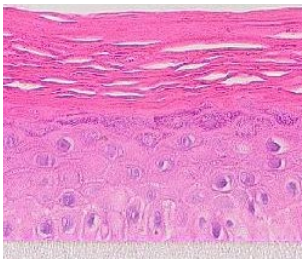
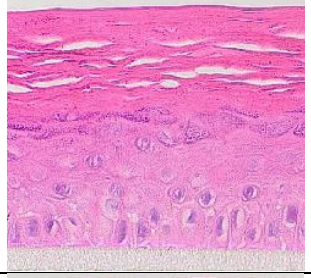
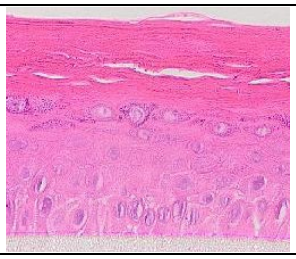
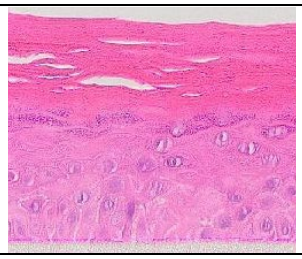
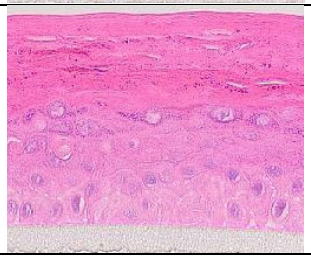
Group	Repeat 1	Repeat 2	Repeat 3
Control group			
Recombinant human type III collagen (CL90) 0.5%			
Recombinant human type III collagen (CL90) 2%			

Table 9 shows that, compared with the control group, the tissue structure of the sample with the administration concentration of 0.5% had clear boundaries, the living cell layers were closely arranged, and the vacuolation phenomenon was significantly improved. When the drug concentration was 2%, the stratum corneum porosity of the samples was improved, the number of viable cell layers was increased, the cells were closely arranged, and the vacuolation was improved.

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Table 10: FLG Immunofluorescence Results

Group	Relative IOD mean	SD	P-value	Lifting rate
Control group	1.00	0.05	/	/
Recombinant human type III collagen (CL90) 0.5%	0.79	0.03	0.005**	19.7%
Recombinant human type III collagen (CL90) 2%	0.81	0.03	0.004**	22.7%

As shown in Table 10, when the concentration was 0.5%, compared with the control group, the FLG content of the sample was significantly increased, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce effects on skin repair. When the dosing concentration was 2%, the FLG content of the sample significantly increased, with the increase rate as high as 22.7%, indicating that it had very significant effect on skin repair.

Table 11: LOR Immunofluorescence Results

Group	Relative IOD mean	SD	P-value	Lifting rate
Control group	1.00	0.04	/	/
Recombinant human type III collagen (CL90) 0.5%	0.84	0.05	0.001**	90.9%
Recombinant human type III collagen (CL90) 2%	0.89	0.08	0.000**	102.3%

As shown in Table 11, when the concentration was 0.5%, compared with the control group, the LOR content of the sample was significantly increased, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce effects on skin repair. When the dosing concentration was 2%, the FLG content of the sample significantly increased, with the increase rate as high as 102.3%, indicating that it had very significant effect on skin repair.

Table 12: TGM1 Immunofluorescence Results

Group	Relative IOD mean	SD	P-value	Lifting rate
Control group	1.00	0.07	/	/
Recombinant human type III collagen (CL90) 0.5%	0.77	0.07	0.001**	113.9%
Recombinant human type III collagen (CL90) 2%	0.83	0.05	0.000**	130.6%

As shown in Table 12, when the administration concentration was 0.5%, compared with the control group, the TGM1 content of the sample was significantly increased, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce effects on skin repair. When the dosing concentration was 2%, the content of TGM1 of the sample significantly increased, with the increase rate as high as 130.6%, thereby having very significant effect on skin repair.

Table 13: Immunohistochemical Results of cd44

Group	Relative IOD mean	SD	P-value	Lifting rate
Control group	1.00	0.03	/	/
Recombinant human type III collagen (CL90) 0.5%	0.59	0.03	0.004**	59.5%
Recombinant human type III collagen (CL90) 2%	0.70	0.04	0.001**	89.2%

As shown in Table 13, when the administration concentration was 0.5%, compared with the control group, the CD44 content of the sample was significantly increased, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce effects on skin repair. When the dosing concentration was 2%, the CD44 content of the sample significantly increased, with the increase rate as high as 89.2%, thereby having very significant effect on skin repair.

3.3. Wrinkle resistance test results

The t groups for detect that content of type I collagen are shown in Table 14[1]. Fibroblasts were inoculated at an inoculation density of 4×10^4 cells/well into 24-well plates and incubated overnight in an incubator (37℃, 5% CO_2). The test article working fluid was configured according to the test groups.

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According to the test groups, when the plate-laying rate of cells in the 24-well plate reached 40%–60%, the drugs were administered in groups with 1 mL per well, and three multiple wells were set in each group. After the completion of dosing, 24-well plates were incubated in an incubator (37℃, 5% CO_2) for 24 h. According to the test groups, the groups that needed to be irradiated were irradiated with UVA at a dose of 30 J/cm^2. After the irradiation, the samples were placed in an incubator (37℃, 5% CO_2) for further culture for 24 h. The cells were fixed with 4% paraformaldehyde for 24 h and then subjected to immunofluorescence test with Collagen I. The results are shown in Table 15.

Table 14: Detection of Type I Collagen Content Test Groups

Group	Sample name	Dosing concentration	Detection model	Detection Indicator	Test method
Control group	/	/	Fibroblast	Collagen I	Immunofluorescence
Sample set	Recombinant human type III collagen (CL90)	0.5%			
		2%			

Table 15: Collagen I Immunofluorescence Results

Group	Relative IOD/ mean cell count	SD	P-value	Lifting rate
Control group	1.00	0.02	/	/
Recombinant human type III collagen (CL90)0.5%	0.75	0.06	0.007**	44.2%
Recombinant human type III collagen (CL90) 2%	0.91	0.08	0.002**	75.0%

As shown in Table 15, the collagen I content of the sample group administered with the concentration of 0.5% was significantly increased as compared with the control group, that is, when the concentration of recombinant human type III collagen was 0.0025%, it was effective for skin wrinkle resistance. When the dosage concentration was 2%, the content of Collagen I in the sample group was significantly increased, and the increase rate was as high as 75.0%, which had a very significant effect on the skin wrinkle resistance.

Table 16: Isolated Skin Tissue Test Groups

Group	Sample name	Dosing concentration	Detection model	Detection Indicator	Test method
Control group	/	/	Isolated skin tissue	Tissue morphology, collagen fibers, Collagen IV	H&E, Masson staining, immunofluorescence
Sample set	Recombinant human type III collagen (CL90)	0.5%			
		2%			

The grouping of in vitro skin tissue testing is shown in Table 16. The freshly obtained skin tissues were immersed in 75% alcohol for 30s and then washed three times with sterile PBS buffer. At the end of the day, the skin was cut into 24±2mm^2 tissue blocks with the epidermis facing up and the dermis facing down, and put into the culture die. Then the culture die was transferred to a six-well plate, and 3.7 mL of culture solution was added into each well for culture in a 5% CO_2 incubator at 37℃, and the solution was changed every day. After 2 days of culture of the isolated skin tissues, irradiation and dosing were started with reference to the test groups and corresponding treatment conditions in Table 16. After continuous irradiation for 4 days, the isolated skin tissues were cultured for a further 3 days during which time no irradiation was performed and only sample administration was performed. The skin tissues after drug administration were fixed in the model with 4% paraformaldehyde, embedded, sectioned and subjected to H&E staining and Masson staining, respectively. The sectioned results were collected and photographed by a microscope and analyzed by Image-Pro®Plus image processing software. The skin tissues used for detection were fixed with 4% paraformaldehyde for 24h, and then immunofluorescence detection of silk fibroin (FLG), loricrin (LOR) and transglutaminase1 (TGM1) content was performed. The tissue morphology results are shown in Table 17, the collagen fiber relative area results are shown in Table 18, and the Collagen IV results are shown in Table 19.

As shown in Table 17, the epidermal viable cell layer of the sample administered at a concentration of 0.5% was significantly thickened when compared with the control group. When the drug concentration was 2%, the living cell layer of the epidermis of the samples was significantly thickened, and the tissue morphology was significantly improved.

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Table 17: Organizational Morphology Results

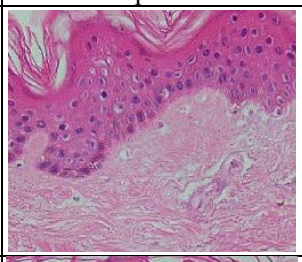
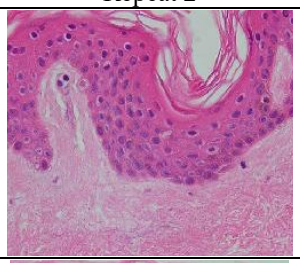
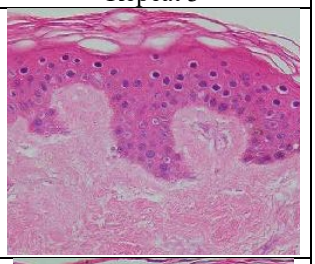
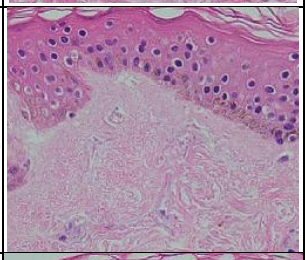
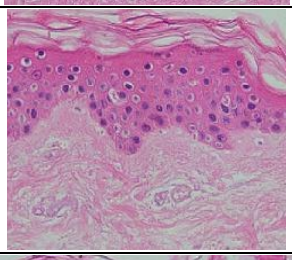
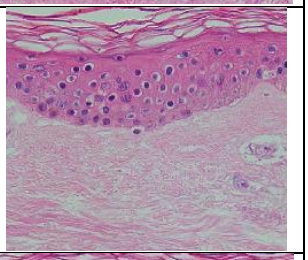
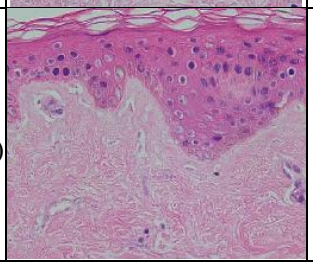
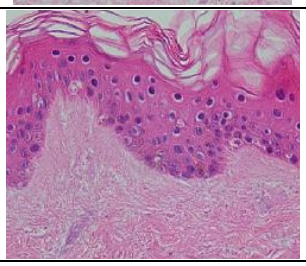
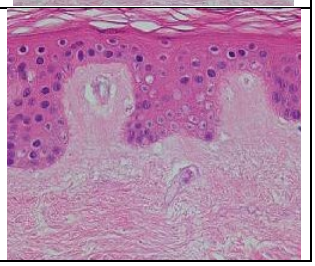
Group	Repeat 1	Repeat 2	Repeat 3
Control group			
Recombinant human type III collagen (CL90) 0.5%			
Recombinant human type III collagen (CL90) 2%			

Table 18: Collagen Fiber Relative Area Results

Group	Average relative area	SD	P-value	Lifting rate
Control group	1.00	0.08	/	/
Recombinant human type III collagen (CL90) 0.5%	0.90	0.04	0.000**	32.4%
Recombinant human type III collagen (CL90) 2%	0.93	0.04	0.000**	36.8%

As shown in Table 18, compared with the control group, the collagen fiber content in the sample group administered with the concentration of 0.5% was significantly increased, that is, when the concentration of recombinant human type III collagen was 0.0025%, it could produce an effect on skin wrinkle resistance. When the drug concentration was 2%, the collagen fiber content of the sample group significantly increased, with the increase rate reaching 36.8%, which had a very significant effect on skin wrinkle resistance.

Table 19: Collagen IV Immunofluorescence Results

Group	Relative IOD/ area mean	SD	P-value	Lifting rate
Control group	1.00	0.01	/	/
Recombinant human type III collagen (CL90) 0.5%	0.76	0.02	0.000**	38.2%
Recombinant human type III collagen (CL90) 2%	0.87	0.03	0.000**	58.2%

As shown in Table 19, the Collagen IV content of the sample group administered with the concentration of 0.5% was significantly increased as compared with the control group, that is, when the concentration of recombinant human type III collagen was 0.0025%, it was effective for skin wrinkle resistance. When the concentration was 2%, the content of Collagen IV in the sample group increased significantly, with the increase rate as high as 58.2%, which showed a very significant effect on skin wrinkle resistance.

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4. Discussion

1) Based on EpiKutis® detection model, the skin water content of recombinant human type III collagen increases at 0.0025%, i.e., it has an effect on moisture retention; when the concentration is 0.01%, the skin water content increases significantly, i.e., the skin has a very significant moisturizing effect.

2) The recombinant human type III collagen at the concentration of 0.0025% can exert certain effects on the relative cell mobility, improvement of tissue morphology, and increase in the content of filament aggregate protein (FLG), loricrin (LOR), transglutaminase1 (TGM1) and hyaluronic acid receptor (CD44). When the concentration is 0.01%, the effect is more significant, i.e., it has a very significant repair effect on skin.

3) At the concentration of 0.0025%, the recombinant human type III collagen can increase the protein content of type I collagen (Collagen I), improve tissue morphology and increase the protein content of collagen fibers and type IV collagen (Collagen IV) to a certain extent, and the effect is more significant at the concentration of 0.01%, i.e., the skin has a very significant anti-wrinkle effect.

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