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Research Article

# Characterization and Application of N,O-Carboxy Methyl Chitosan Produced at Different Tempererature of Etherification

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**ABSTRACT**

The aim of this research is to know the effect of etherification temperatures on characteristics of N,O-carboxymethyl chitosan (N,O-CMC) and to know the healing effect of N,O-CMC ointment against burns in white mice. Derivatization of N,O-CMC was done by reacting chitosan compound with mono chloroacetic acid at different etherification temperatures i.e. 60, 75 and 90ºC for 3 hours. The results indicated that etherification temperatures at 90ºC have the best characteristics with 87.33±2.52 % in solubility, 209.50±0.50% in yield, 0.86±0.02 in degree of substitutions and the appearance of a functional group of -COO- at 1411.89 $cm^{-1}$ and 1604.77 $cm^{-1}$ wavelength. Etherification temperatures that produced the best characteristics of N,O-CMC was used to prepare N,O-CMC ointment to be added on vaseline album as the ointment base with 5% concentration. The effectiveness of burns healing by N,O-CMC ointment would be compared with other treatment namely Chitosan ointments 5%, Vaseline album ointment (negative control) and commercial ointment (positive control). Statistical analysis results indicated that N,O-CMC ointment has the best activities in white mice burns healing compared to three other treatments. N,O-CMC ointment was able to heal the burn of white mice in 32±1 days.

**Keywords**: burns, chitosan, N,O-carboxymethyl chitosan, ointment.

## INTRODUCTION

Chitosan is a compound with chemical formula of poly 2-amino-2-dioksi-ß-D-glucose. It can be produced by the hydrolysis of chitin using a strong base, this process called deacetylation[1]. According to Koevet al.[2], chitosan, as a biomaterial, is suitable to be developed and applied in many fields due to its biocompatible and biodegradable properties. In addition, degradation products of chitosan are harmless and non-antigenic (do not cause an immune response in an organism). Thus, chitosan is commonly used in a fabrication (fibroblast formation) in particular as a thread, cover and substrate materials that are biodegradable to human skin epithelial growth[3]. One of the problems in the application of chitosan is its low solubility in water. Chitosan is only soluble in acid (usually in a weak acid) and their solubility decreases with increase in pH[4]. This is an obstacle in the widespread utilization, especially in the pharmaceutical industry, cosmetics, and food, as many product applications of these industries that require the use of chitosan which is soluble in water. Modification of chitosan increases its solubility in neutral pH. Zhang et al.[5] added o-succinyl groups to increase the solubility of chitosan in aqueous media. Hayes[6], reacting chitosan with mono chloroacetic to produce N,O-carboxymethyl chitosan (N,O-CMC) that are soluble in water. Basmal et al.[7] stated that the etherification temperature at CMC derivatization processes affects the quality and quantity of produced CMC. The solubility and yield of N,O-CMC increases by the increasing of etherification temperature. Based on their properties, chitosan, and its derivatives can be used as water-soluble pharmaceutical products. PataleandPatravale[8] said that increasing of electric charge caused the chitosan easily soluble in water. Higher solubility in water improves the bioactivity of chitosan and its derivatives, such as antimicrobial activity and their reactivity in wound healing treatment. The research was conducted to examine the use of N,O-CMC as wound healing material in the ointment and prove that higher solubility of chitosan can improve its activity in wound healing treatment.

## MATERIALS AND METHODS

*Materials and instrumentation*

The materials were used for derivatization and characterization of N,O-CMC including chitosan from freshwater prawns (*Machrobachium rosenbergii*), isopropanol, sodium hydroxide (NaOH), monochloro acetic acid, distilled water, methanol, acetic acid glacial, hydrochloric acid and ethyl chloride.The instruments used for derivatization and characterization of N,O-CMC including hot-plate stirrer, glassware, thermometers, gauze filter, oven, FT-IR Spectrometer Perkin Elmer System 2000 (Shimadzu).

*Derivatization of N,O-CMC*

The derivatization of N,O-CMC was performed using the method of Hayes[6] with modification of carboxylation/etherification temperature, in which 60, 75 and 90ºC were used in this study. A total of 2 g of chitosan

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(DD 92.75%) was dissolved in 20 ml of isopropanol and stirred at room temperature until blended and 10 M NaOH was further added about 5 mL slowly for 45 minutes. The next step was the addition of 2.4 g monochloro acetic acid for 20 minutes. Etherification process was carried out at a temperature range 40-70ºC for 3 hours. The next process was the addition of 1.6 mL of cold distilled water and then the pH was adjusted to 7 with glacial acetic acid. After reaching a neutral pH, the solution was filtered and the solids were washed with 70%methanol with a ratio of 1:15. Then a second washing was performed using anhydrous methanol with the same ratio. The solids were taken and dried in an oven with a temperature of 60°C for 24 hours. N,O-CMCproduced from derivatization process were then characterized. Parameters that were tested including the solubility, yield, degree of substitution[9], and functional groups using FT-IR (Fourier Transform Infra Red)[10].

*Aplication of N,O-CMC*

The best results from the characterization of N,O-CMC was used as a raw material ointment base with vaseline album. A total of 5% ointment N,O-CMC of the best etherification temperature treatment was compared with other treatments namely chitosan 5% ointment, ointment vaseline album (negative control) and commercial ointment (positive control). This study used 12 male rats Sprague Dawley strain, ±2 months of age and weighing 300-400 g. Burns was induced on the skin of the back using heat at a temperature of 180°C for 5 seconds. The last process was to perform a variety of ointment administration treatments in rats on a daily basis. The degree of healing was examined periodically by measuring the ratio of wound diameter after and before treatment. Data were statistically analyzed using Complete Randomized Design performed by SPSS ver. 9.

## RESULTS AND DISCUSSION

*Effect of etherification on solubility*

Solubility is a characteristic of N,O-CMC. The solubility of N,O-CMC produced with etherification temperature of 60, 75, and 90ºC were 75.33±2.08, 80.00±2.65, and 87.33±2.52%, respectively. It showed that the solubility increases with the increasing of temperature in the etherification process as presented in Table 1. There was no significant difference between the results of the solubility at 60°C and 75ºC, but the data indicated a significant difference between treatments at a temperature of 90ºC and 60ºC also75ºC and 90ºC. The results showed that there were increasing values of N,O-CMC solubility (87.33±2.52%) when compared with the initial chitosan solubility (11.67%; w/v)[11]. This condition proved that by reacting chitosan with monochloro acetic to N, O-CMC increased its solubility.[6] The solubility of N,O-CMC from each treatment also increased due to the increase in temperature etherification. This condition, as stated by Basmal et al.[7], revealed that the etherification temperature influences on the solubility value. Further Basmal et al.[7] explained that the solubility value was strongly influenced by the number of carboxymethyl groups that react with the chitosan chain atoms $C_2$ and $C_6$. A growing number of carboxymethyl groups that react will increase solubility, however, the temperature was important in substituting carboxymethyl groups into chitosan

*Effect of etherification on yield of N,O-CMC*

The yield of N,O-CMC at different temperature (60, 75, and 90ºC) was 185.00±2.29, 207.17±1.04, and 209.50±0.50%, respectively. There were increasing in yield at each temperature (Table 1), indicating that the treatment temperature affects the yield. The yield of N,O-CMC is the result of the substitution of $H^+$ on the $C_6$ atom of $CH_2COO^-$. Thus, the greater the value of yield, the more substitution that occurred in the N,O-CMC compound. According to Basmal et al.[7], etherification temperature increase allows the carboxyl group, not only attached to the $C_6$ atom but also reacting with the $C_2$ atom in the amine group. The yield of N,O-CMC prepared in this study is larger compared to Basmal et al.[7] who use the same temperature. This differences may occur due to differences in methods of derivatization of N,O-CMC or could also be differences in raw material chitosan where Basmal et al.[7]using a small crab chitosan raw material whereas in this study using prawn chitosan. This yield value is directly proportional to the solubility value, meaning that the higher the yield, the higher the solubility. This condition possibly occurs as both parameters, yield and solubility, are affected by the amount of monochloro acetic carboxyl group bonded to the chitosan compound.

*Effect of etherification on degree of substitution*

The degree of substitution (DS) is the number of substituted groups in the chitosan monomer. Hayes[6] explains that the value of DS is 1.0 and 0.0. The value of 1.0 means that there is one group in chitosan monomer substituted and the value of 0.0 means that no group substituted, but in the polymer are certainly not all perfect substituted monomers. In the polymer of N,O-CMC, DS maximum and minimum value were 2.0 and 0.0 which indicates that no carboxymethyl group substituted. The results showed that the treatment temperature of 60°C and 75°C had significant differences while the treatment temperature 75°C and 90°C were not significantly different. Descriptively, treatment temperature affects the increase in the value of DS. DS values increasing with increasing temperature treatment etherification as presented in Table 1. Hayes[6] shows that the DS value of N,O-CMC of 0.4 to 0.8 resulted from etherification temperature of 40ºC to 70ºC. The results of this study were included in the DS range used by Hayes[6], in which treatment temperature of 60°C resulted to a DS value of 0.76. Hayes also explained that the value of the DS are small due to the difficulty in substituting 2 carboxymethyl groups simultaneously and continuously. A similar study conducted by Basmal et al.[7] obtain different results in this study. The DS resulting in the study with etherification temperature of 60, 75, and 90ºC was 1.46, 1.29, and 1.18, respectively. DS values in this study tended to decrease with increase in temperature. This condition can occur because the polymer chains that exist in the N,O-CMC clipped along with the increase in temperature. The increase in temperature is accompanied by an increase in the value of the DS showed that temperature has an important role in determining the value of DS. The higher

Table 1: Characteristics of N,O-CMC prepared by different etherification temperatures.

| Temperature | Solubility (%) | Yield (%) | Substitution degree |
|---|---|---|---|
| 60ºC | 75.33±2.08[a] | 185.00±2.29[a] | 0.76±0.03[a] |
| 75ºC | 80.00±2.65[a] | 207.17±1.04[b] | 0.83±0.03[b] |
| 90ºC | 87.33±2.52[b] | 209.50±0.50[b] | 0.86±0.02[b] |

Note: Signs different letters in the same column indicate significant difference ($p < 0.05$)

Table 2: The functional group of N,O-carboxymethyl chitosan.

| Fuctional group | Silverstein et al.[12] | Stuart[13] | Erna et al.[10] | Etherification 60ºC | Etherification 75ºC | Etherification 90ºC |
|---|---|---|---|---|---|---|
| C-O-H | 1424 $cm^{-1}$ | 1430 $cm^{-1}$ | 1416 $cm^{-1}$ | 1411 $cm^{-1}$ | 1404 $cm^{-1}$ | 1411 $cm^{-1}$ |
| C=O | 1850-1600 $cm^{-1}$ | 1680-1660 $cm^{-1}$ | 1606 $cm^{-1}$ | 1620 $cm^{-1}$ | 1627 $cm^{-1}$ | 1604 $cm^{-1}$ |
| N-H | 3497-3077 $cm^{-1}$ | 3335 $cm^{-1}$ | 3437 $cm^{-1}$ | 3448 $cm^{-1}$ | 3425 $cm^{-1}$ | 3448 $cm^{-1}$ |
| O-H | 3800-2700 $cm^{-1}$ | 3600 $cm^{-1}$ | 3437 $cm^{-1}$ | 3749 $cm^{-1}$ | 3749 $cm^{-1}$ | 3749 $cm^{-1}$ |

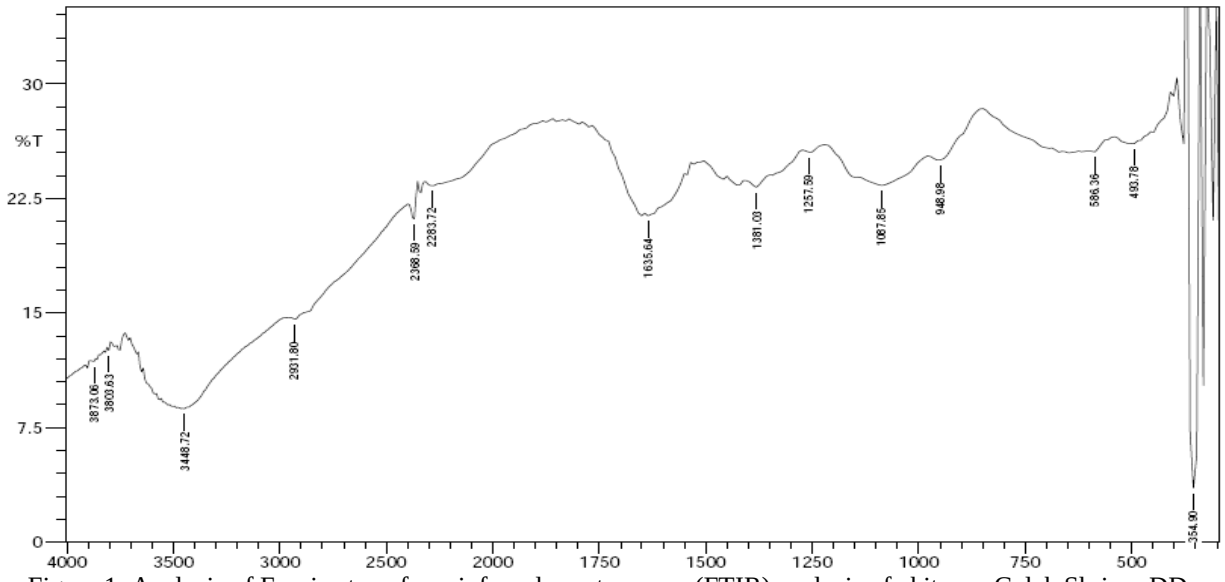


Figure 1: Analysis of Fourier-transform infrared spectroscopy (FTIR) analysis of chitosan Galah Shrimp DD 92.75%[13].

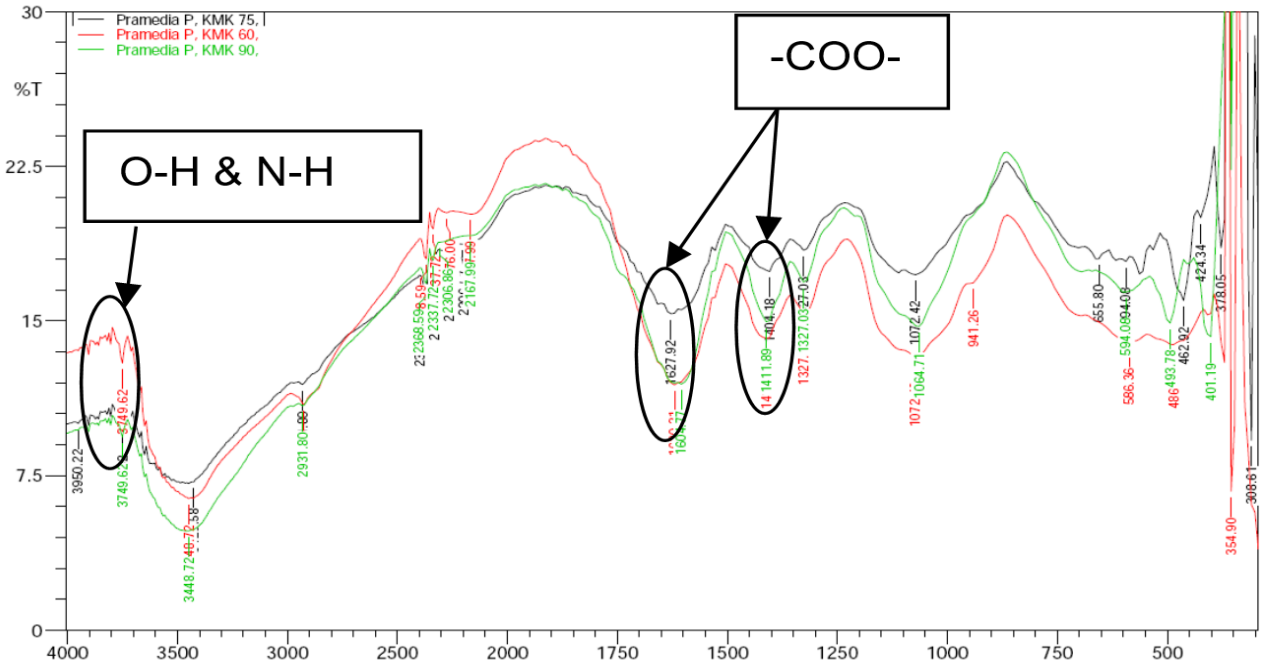


Figure 2: FTIR spectra of N,O-CMC overlap, etherification temperature of 60, 75, and 90ºC ($cm^{-1}$).

value of DS indicates that more number of carboxymethyl groups (-$CH_2COOH$) substituted in the polymer chitosan.

*Effect of etherification on functionalgroups*

FTIR analysis of N,O-CMC showed that there were differences between IR spectrum of chitosan (Figure 1) and N,O-CMC (Figure 2). The most visible difference was the appearance of a new peak at a wavelength of 1404.18 to1411.89 $cm^{-1}$ together with a peak at 1604.77 to 1627.92 $cm^{-1}$ form distinctive groups that exist in the N,O-CMC ie.$^{-}$COOH group. Carboxymethyl chitosan has several types of atomic bonding which are N-, O-, N-O, and N-N carboxymethyl chitosan. Figure 2 shows that the peak in range 3425.58 to 3448.72 $cm^{-1}$ was sharper when compared with a peak of chitosan. This condition occurs because the $H^+$ ion substitution by the carboxyl group of bonds vibrate at these wavelengths was-N-H bond. While at a wavelength of 3749 $cm^{-1}$ there is a peak of O-H functional group. These conditions provide a description that carboxymethyl chitosan on this treatment was N,O-CMC the carboxyl group attached to the -O- and -N= or commonly referred to as N,O-carboxymethyl chitosan. Similar results spectrum of N,O-CMC was reported by Erna et al.[10]. Etherification at 90°C has a very sharp peak at -OH and -$NH_2$ which binds to carboxyl group were shown in wavelength 3448.72 $cm^{-1}$ and 3749 $cm^{-1}$, while the sharpness was reduced at 60 and 75ºC with a wavelength of 3448.72 and 3425.58$cm^{-1}$, respectively. Erna et al.[10]stated that the sharpness of spectrum peak associated with the substitution of the carboxyl group. The optimum value of -COO- functional groups bond shown in two wavelength range that was 1404.18 to 1411.89$cm^{-1}$ and 1604.77 to 1627.92 $cm^{-1}$. Etherification at 90 and 60ºC have an optimum peak in the range of peak 1604.77 to 627.92 $cm^{-1}$, followed by 70°C, while at 60°C has an optimum peak in the range of wave 1404.18 $cm^{-1}$ to 1411.89 $cm^{-1}$ were successively followed by 90ºC and 75ºC etherification treatment. Etherification treatment at 60°C was a difference with the two other treatments that led to peak at a wavelength of 941.26 $cm^{-1}$. According to Stuart[12] vibration occurs at a wavelength of 1250–900 $cm^{-1}$ was a CO group of ether compounds, the possibility of this compound is no less than perfect due to leaching by methanol compound. Base on the yield, solubility, degree of substitution and functional groups showed that etherification at 90°C was the best condition.

*Application of N,O-CMC ointment on burns*

Figure 3 showed the result of the application of various ointment preparations on the burns-induced wound of the rat with initial wound diameter of 3 cm, the calculation according to Suratman et al[14]. It indicates that the degree of healing increased along with the treatment of ointment application period, except for the negative control treatment. Treatment of negative control on the second day to the fifth day resulted into the negative average value, indicating the deviation of the wound healing process. Negative values implied that the wound diameter is increasing. Infection of the wound may cause the diameter magnification, but the condition did not last long. The infection takes place only at the beginning of the manufacture of burns and early treatment. This incident proved that the other three treatments, namely chitosan, N,O-CMC and commercial ointment have the ability to prevent infection. The results showed that chitosan, N,O-CMC and commercial ointment has properties that should be possessed by the wound healing compound, specifically to prevent the entry of the wound pathogens that can cause infections. This result was similar to the statement that the N,O-CMC act as biophysical, a physical barrier against surface overlaid[15]. This coating ability explained why the infection of wound did not occur in mice treated with

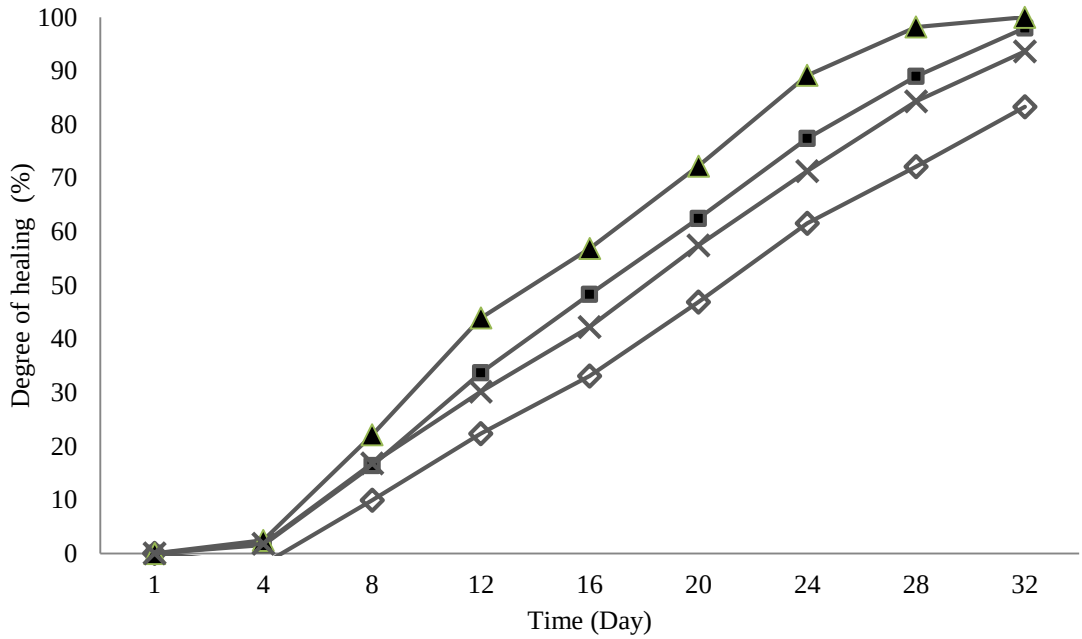


Figure 3: The influence of ointment type (◊=ointment of vaseline album, ■=ointment of 5% chitosan ▲=ointment of 5% N,O-CMC, ×=commercial ointment) on the rate of burn healing of white mice.

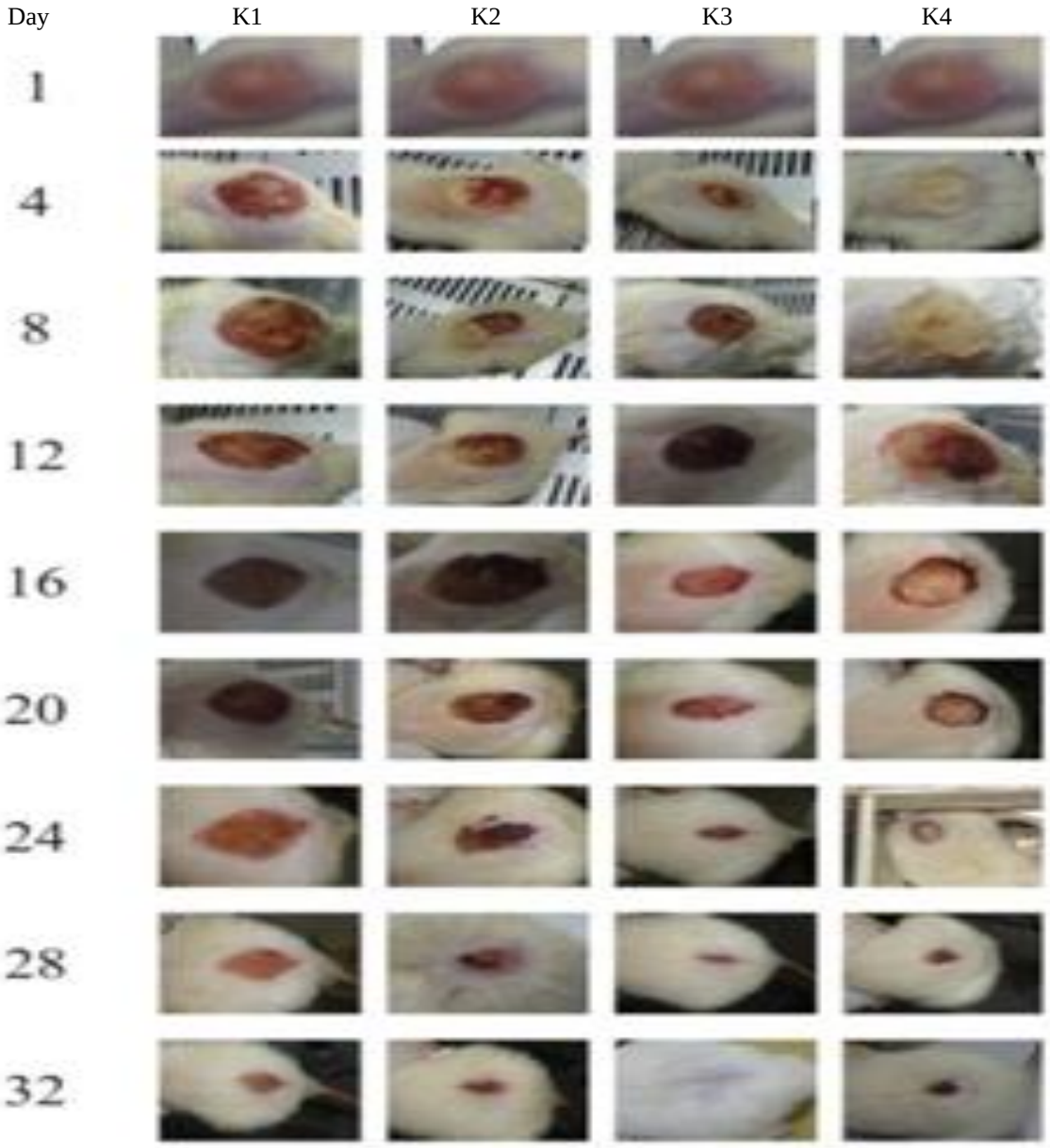


Figure 4: The process of development of burn healing in mice with different treatments. Description: K1=Negative control (vaseline album 100%); K2=Ointment chitosan (concentration 5%); K3=Ointment N,O-CMC (concentration 5%); K4=Positive control (Bioplacenton®).

chitosan, N,O-CMC and commercial ointment (Figure 4). The results showed that N,O-CMC in the form of an ointment with a concentration of 5% can heal wound faster than chitosan ointments(5%) and Bioplacenton® album Vaseline ointment base. The results showed that the ointment of N,O-CMC has the ability of healing burns better than ointment chitosan. According to Muzzarelli[16], changes in biocompatibility, improved biodegradability, low toxicity, an increase in antimicrobial activity and moisture content of N,O-CMC, making it an excellent biomaterial for wound healing. A similar statement also expressed by Chen et al.[17], which proved that the N,O-CMCis able to improve bioactivity in skin fibroblasts by 2-fold, stimulates proliferation in normal skin fibroblasts and prevent the proliferation of keloid skin fibroblasts. N,O-CMC served to stimulate extracellular lysozyme activity in stimulating the proliferation of fibroblasts and skin fibroblasts. Derivatization of N,O-CMC with temperature differences (60, 75, and 90ºC) in etherification was showed a marked influence on yield parameters, solubility, degree of substitution and functional groups. Treatment derivatization of N,O-CMC with a temperature of 90°C was the best treatment of etherification base on parameters of yield (209.50 ± 0.50%), solubility (87.33 ± 2.52%), degree of substitution (0.86), and functional group with a typical peak of N,O-CMC at a wavelength of 1411.89 and 1604.77 $cm^{-1}$. Healing ointment treatment with N,O-CMC was the best ointment treatment that was able to heal the wounds in 32±1 days.

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