Effect of Concentration Differences of Snail Mucus Gel (Achatina Fulica) on Collagen Density and Wound Closure Rate in Wistar Rat Skin Punch Biopsy Wounds

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Abstract

Collagen production and deposition is crucial to replace damaged tissue. Glycosaminoglycans in snail mucus has high biological value for wound healing. This study aims to determine the effect of concentration differences of snail mucus gel on collagen density and wound closure rate in skin punch biopsy wounds. Quasi experimental research was done using 6 Wistar rats, each wounded with 4 punch biopsy wound in dorsa skin, and subsequently applied with 24%, 48%, 96% snail mucus gel and 3% CMC-Na gel. Collagen density was measured on 2nd and 7th day using histophotometry analysis. Wound closure rate was measured from wound area on the 4th and 7th day.Data analysis using 2-way Anova showed significant differences in collagen density ($p=0.000$) and wound closure rate ($p=0.000$) of all treatment groups and observation period, but there is no interaction between them. Post hoc LSD showed significant difference in wound closure rate between control groups and all treatment groups ($p=0.000$), but there is no significant difference between treatment groups ($p>0.05$). Significant differences of collagen density was found between controlgroups and all treatment groups ($p>0.05$). Significant difference of collagen density between treatment groups only found on snail mucus gel 96% ($p<0.05$).In conclusion, topical application of snail mucus gel can increase collagen density and wound closure rate in Wistar rat skin punch biopsy wound healing. Wound closure rate was not influenced by concentration differences of snail mucus gel, although the collagen density proved to be much higher in the 96% snail mucus gel group.

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Introduction

Management of soft tissue injuries with satisfactory results functionally and aesthetically is a challenge for Oral and Maxillofacial Surgeon[1,2]. Various attempts were made to be able to get optimal wound healing, including the application of dressing material, organic material, medicines, to environmental manipulations such as temperature and oxygenation[3].

Snail mucus has been extensively researched to help accelerate wound healing[4], containing at least four essential natural elements that are good for the skin, namely alantoin, antimicrobial peptides, enzymes, and glycoproteins[5,6,7]. Utilization of snail mucus requires further research to scientifically prove its efficacy claim before it can be used widely[8].

Snail mucus has been heavily integrated with various drug carrier such as alginat-carboxymethyl cellulose (alginat-CMC)[9], carboxymethyl cellulose sodium salt (CMC-Na)[10], plasticizer polyethylene glycol 400 (PEG 400)[11], and cream[12]. Existing research suggests snail mucus can work more effectively when integrated with drug carrier and there is a relationship between snail mucus concentration and its wound healing effect[9,10,11,12]. The minimum concentration of an animal product with optimal working effect needs to be revealed to maintain its ecological aspect[13].

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Wound healing process rely on correct collagen metabolic process starting from the regulation of collagen production, deposition, and maturation. Evaluation of collagen formation becomes an important parameter to evaluate wound healing[14]. Collagen analysis can be performed biochemically, histologically, and clinically. Histological staining is able to provide clearer information regarding the organizational structure of the formed collagen fibers. Along with the deposition of collagen replacing damaged skin structure, the wound will get smaller and smaller until it disappears clinically. Wound closure rate can be observed macroscopically and quantitatively by measuring the wound area on each observation day. Wound closure rate can be a clinical indicator to assess product potency in accelerating wound healing15.

This study discusses the effect of concentration differences of snail mucus gel application on collagen density and wound closure rate in skin punch biopsy wound healing. Interaction between concentration and length of observation days will also be observed.

Materials and methods

This study had obtained ethical clearance from Ethics Commission of the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta (No. 00273/KKEP/FKG-UGM/EC/2019). Six Wistar rats that met the inclusion criteria were adapted and four punch biopsy wounds with diameter of 5mm were created on the dorsal region of each rat. Wound sample on the dorsal region were divided into 4 groups, namely *3%* CMC-Na gel, 24% snail mucus gel, 48% snail mucus gel, and 96% snail mucus gel. The amount of 0.1 ml of gel was applied to the wound surface every 24 hours. Six rats were divided into 2 groups based on decapitation day, namely 3 rats on the 2nd day and 3 rats in the 7th day. Collagen density was measured using slide from day 2 and 7. The rat decapitated on day 7 were observed clinically on day 4 and 7 to measure the wound closure rate.

Collagen density was observed using Mallory stained slide. Observation was done using light microscope with 40x magnification first to identify wound area. Five fields of view were selected with a magnification of 400x from each slide, provided that all five fields of view were located in the reticular layer of the dermis in the wound area. Photographs were taken from all five fields of view. The image is then processed with imageJ v1.52a application to isolate the blue area using color deconvolution feature. The blue spectrum image is then processed using treshold feature to mark the collagen fiber. The process produce a binary image that can be used to quantify percentage of observed area. Collagen density is expressed as the percentage of the blue area in the observation field that was processed using imageJ application. Collagen fiber density data is a mean of 5 different fields of view.

Wound closure rate was observed clinically on day 4 and 7 after *punch biopsy* procedure. Measuring tool using milimeter block paper with a hole is placed over the wound, then the wound surface is photographed with a digital camera. The image is then processed using imageJ application. Draw a straight line connecting two points in the image whis is known to be 5mm long. Set the measurement scale (set scale) by comparing the size of the pixel length of the straight line with the actual known length of 5mm. The wound edge is traced using freehand tool and wound area is measured in cubic milimeters (mm^2). Wound closure rate is expressed as percentage of wound area compared with that on postoperative day according to the following formula:

$$\text{Wound closure rate (\%)} = \frac{\text{initial wound area - wound area at each visit}}{\text{initial wound area}} \times 100\%$$

Results

All rats were in good health and physically active during the study. No rats died and there was no sign of wound infection. Rats body weight were stable between 250-300 grams and met the inclusion criteria.

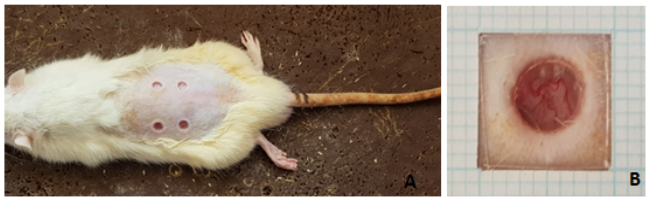

Figure 1. Wistar rat after punch biopsy procedure. 4 excision wounds on the dorsal area (A). The initial excision wound with a diameter of

5mm was observed using a measuring grid (B).

1. Collagen Density

Observation initiated with low magnification of 40x to find punch biopsy wound area(Figure 1). The overall wound area was well observed, the wound margins with healthy tissue were clearly visible, the scab was obvious, and the papillary and reticular stratum of the wound was clearly visible (Figure 2 and 3).

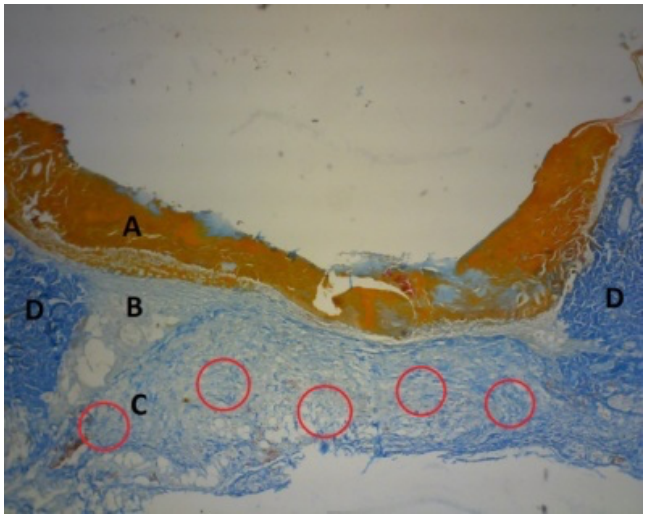

Figure 2. Wound healing process on day 2 on Mallory slide at 40x magnification showed scab (A), papillary stratum (B), reticular stratum (C), and healthy dermis at the wound margins (D). The observation area at 400x magnification is marked with a red circle.

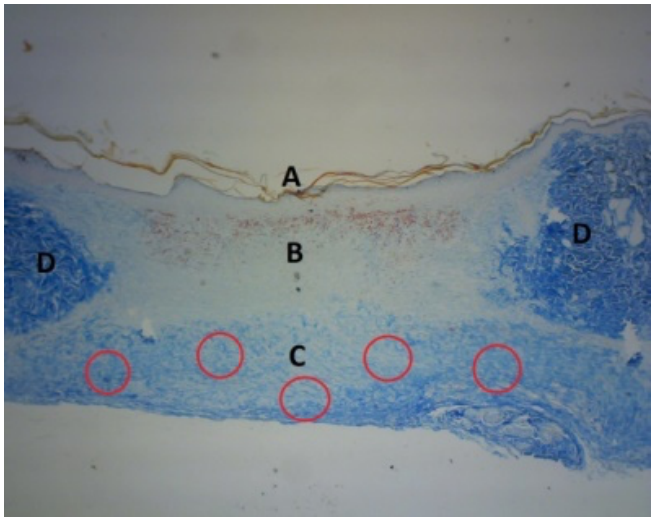

Figure 3. Wound healing process on day 7 on Mallory slide at 40x magnification showed scab (A), papillary stratum (B), reticular stratum (C), and healthy dermis at the wound margins (D). The observation area at 400x magnification is marked with a red circle.

The wound on day 2 showed a thick scab (Figure 2A). Collagen fibers in the wound area have begun to form with multidirectional fiber orientation and appear sparse (Figure 2C). Collagen fibers formation appears to be more dominant in reticular stratum (Figure 2C) than in the papillary stratum (Figure 2B). The wound on day 7 showed a thinning scab (Figure 3A). More collagen fibers can be observed in the reticular stratum with higher density than day 2 (Figure 3C).

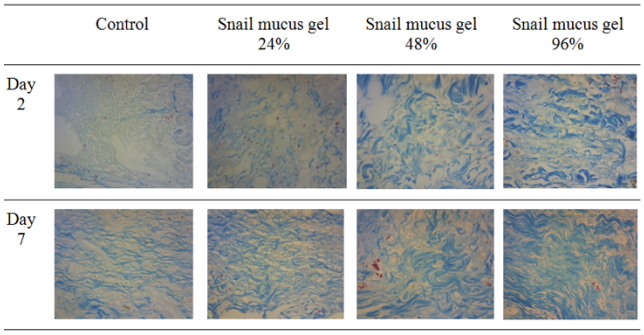

Figure 4. Microscopic image of collagen density with Mallory staining (400x magnification) on Wistar rat skin punch biopsy wound healing.

Comparison of collagen density at 400x magnification showed an increase in collagen density from day 2 to 7 in all treatment groups (Figure 4). The image was processed using imageJ application (Figure 5) to obtain collagen density data (Table 1).

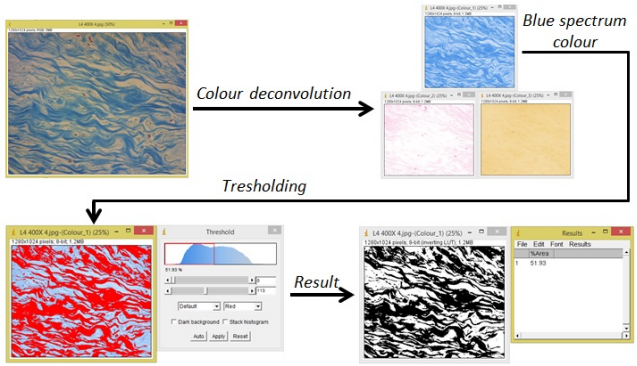

Figure 5. Image processing using imageJ to obtain collagen density data.

Observation day	Mean ± SD (%)			
	Control (N=3)	Snail mucus gel 24% (N=3)	Snail mucus grl 48% (N=3)	Snail mucus gel 96% (N=3)
Day 2	18.47 ± 4.92	27.36 ± 1.76	31.34 ± 2.30	35.71 ± 2.89
Day 7	38.77 ± 2.42	40.18 ± 4.89	42.88 ± 0.60	48.81 ± 5.29

Table 1. Mean and standard deviation (SD) of collagen density (%) on each treatment group

based on observation day.

Observation day	Mean ± SD (%)			
	Control	Snail mucus gel 24%	Snail mucus gel 48%	Snail mucus gel 96%
Day 4	14.51 ± 1.47	34.89 ± 3.29	35.06 ± 4.46	41.46 ± 1.26
Day 7	61.93 ± 5.10	71.21 ± 6.67	72.86 ± 9.97	74.07 ± 6.08

Table 2. Mean and standard deviation (SD) of wound closure rate (%) on each treatment group based on observation day.

The data were normally distributed (p=0.200) and homogeneous (p=0.100). The 2-way ANOVA test for the treatment group showed a significant difference (p=0.000). The observation day group also showed a significant difference (p = 0.000), but no interaction was found between them (p = 0.169). The post hoc LSD test (Table 2) showed that all treatment groups had a significantly higher collagen density than the control group. The highest collagen density was found on 96% concentration which differed significantly with 24% and 48% concentrations.

2. Wound closure rate

Wounds in all groups were reduced in size from day 4 to day 7, clean, covered with scabs, and none had infection (Figure 6). Wound area was measured using ImageJ and processed to obtain wound closure rate data (Table 2).

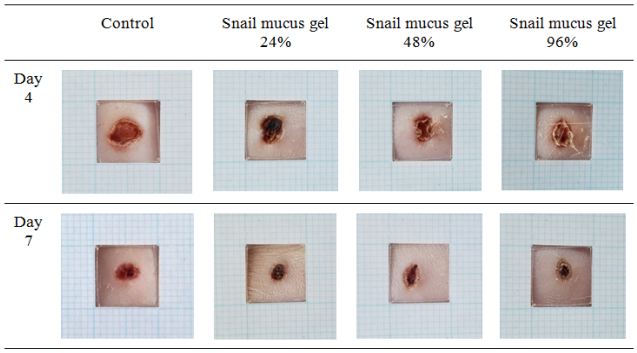

Figure 6. Clinical photographs depict wound closure rate on Wistar rat skin punch biopsy wound healing.

The data were normally distributed (p = 0.357) and homogeneous (p = 0.056). The 2-way ANOVA test for the treatment group showed significant differences (p = 0.000). The observation day group also showed a significant difference (p = 0.000), but there was no interaction between them (p = 0.158). Post hoc LSD test showed that all treatment groups had significantly higher wound closure rates than the control group, but there was no significant difference in wound closure rates between treatment groups (24%, 48%, and 96%).

Discussion

This study aims to determine the effect of concentration differences of snail mucus gel on collagen density and wound closure rate in skin punch biopsy wound healing. Secondary dermal wound healing involves a complex physiopathological process due to significant loss of tissue substance, so the analysis of secondary healing processes in response to various forms of dermal substitution is important to study efficient therapeutic products to stimulate wound healing[16,17,18].

Snail mucus has been extensively researched and proven to be beneficial for wound healing[4]. The use of pharmaceutical materials as a drug delivery system for snail mucus has also been widely studied and proven to increase the effectiveness of snail mucus in helping accelerate wound healing[9,10,11,12]. This study used CMC-Na 3% as a gelling agent for snail mucus[10,19]. CMC-Na binds the snail mucus into its structure, keeps it stable, does not sediment, and helps the substance adhere to the wound surface. The optimal adhesion of snail mucus gel to the wound surface allows the active ingredients of the snail mucus to be absorbed longer, delivered consistently, and work more effectively in helping the wound healing process[10].

Measurement of new collagen formation and its quality is important in animal skin wound healing studies. Collagen density in this study was calculated using histophotometric analysis. This method is quite simple, fast, inexpensive, and can provide satisfactory and realistic results[14].

All groups showed a significant difference in collagen density between day 2 and day 7 (p = 0.000). This result is in accordance with the results of research by Caetano et al[14] regarding normal physiology of Wistar rats skin wound healing, which showed that collagen was observed on day 2 and its deposition continued to increase until a higher collagen density was obtained on day 7.

Significant differences were found between the control group and the snail mucus gel group of 24% (p = 0.022), 48% (p = 0.001),

and 96% (p = 0.000). These results indicate that the CMC-Na gel which is loaded with snail mucus can accelerate the proliferation phase, so that the collagen density in the snail mucus gel group is generally higher than the control group.

The 96% snail mucus concentration had the highest mean of collagen density in day 2 (35.71% ± 2.89) and 7 (48.81% ± 5.29), and was significantly different from the control group (p = 0.000), 24% concentration (p = 0.001), and 48% concentration (p = 0.022). Snail mucus gel 96% proved to be the best concentration to increase collagen density. These results are consistent with the findings of Rahmawati et al[9] and Gunawan et al[20] which stated that the more mucus content of the Achatina fulica snail, the faster the wound healing was. Efforts to accelerate wound healing generally focus on shortening the inflammatory phase and accelerating the proliferation stage[17]. Optimal collagen formation in the initial wound healing phase will greatly help speed up the wound healing process[14].

Snail mucus is rich in protein related to the biochemical synthesis of collagen molecules. Snail mucus contents that can affect fibroblast proliferation include heparin and heparan sulfate. Heparan sulfate helps speed up the wound healing process by assisting blood clotting process and fibroblasts proliferation. Heparan sulfate as a proteoglycan is able to bind and store bFGF. This growth factor is released into the extracellular matrix and stimulates the recruitment of inflammatory cells, activation of fibroblasts, and formation of blood vessels. The additional concentration of heparan sulfate absorbed by the tissue will increase the proliferation of fibroblasts[12]. Heparin is able to increase the stability of fibroblast growth factor 1 (FGF1) and determine the active complex of fibroblast growth factor 1-fibroblast growth factor receptor (FGF1-FGFR). FGF1 plays an important role in regulating fibroblast, endothelial and epithelial cell proliferation and influences angiogenesis through endothelial cell activitation[21].

Increasing amount of heparan sulfate and heparin plays an important role in the initial phase of wound healing. Naturally, heparan sulfate and heparin are found in acute wound fluid within 24-72 hours after injury, bind to heparin binding growth factor (HB-EGF), which can act as a mitogenic agent for fibroblasts, smooth muscle cells, and epithelial cells. The higher the snail mucus content, the higher the additional heparan sulfate and heparin concentrations that will be absorbed by the tissue. The addition of heparan sulfate and heparin will increase the activation and proliferation of fibroblasts, which leads to an increase in collagen density.

Increased collagen deposition in the wound area is generally related to the faster wound healing process. Wound healing can be observed clinically by closure of the wound area. The faster the wound closes, the faster the wound healing process occurs. This study showed a significant difference in wound closure rates in the snail mucus gel group of 24% (p = 0.000), 48% (p = 0.000), and 96% (p = 0.000) compared to the control, although there was no significant difference between the snail mucus gel 24% with 48% (p = 0.778), 48% with 96% (p = 0.248), and 24% with 96% (p = 0.157).

The mean of wound closure rate of 96% snail mucus gel group was 41.46% ± 1.26 in day 4 and 74.07% ± 6.08 in day 7. This mean was higher than the 24% snail mucus gel group (34.89% ± 3.29 in day 4 and 71.21% ± 6.67 in day 7) and 48% snail mucus gel group (35.06% ± 4.46 in day 4 and 72.86% ± 9.97 in day 7), although thus this difference was not statistically significant. The significantly higher collagen deposition in 96% snail mucus gel group did not necessarily make the wound closure rate faster than the 48% and 24% snail mucus gel groups. The insignificant difference between groups of 24%, 48%, and 96% showed that the three concentrations had the same ability to increase the clinical wound closure rate, although the 96% snail mucus gel concentration had the advantage of stimulating higher collagen production significantly.

Conclusions

Topical application of snail mucus gel can increase collagen density and wound closure rate in Wistar rat skin punch biopsy wound healing. Wound closure rate was not influenced by concentration differences of snail mucus gel, although the collagen density proved to be much higher in the 96% snail mucus gel group.

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Declaration of Interest

The authors report no conflict of interest.

References

1. Kementerian Kesehatan Republik Indonesia. Hasil Riskesdas 2018. Jakarta: Kementerian Kesehatan Republik Indonesia; 2018: 127.
2. Braun TL, Maricevich RS. Soft Tissue Management in Facial Trauma. Seminars in Plastic Surgery. 2017; 31(2): 73-9.
3. Phillips SJ. Physiology of Wound Healing and Surgical Wound Care. ASAIO Journal. 2000; 46: 2-5.
4. Prasojo S, Rahajoe PS, Hasan CY. Efek Pemberian Lendir Bekicot (Achatina fulica) terhadap Peningkatan Angiogenesis pada Luka Sayat Kulit Mencit (Mus musculus) (Kajian Imunohistokimia CD34). Thesis: Universitas Gadjah Mada; 2018.
5. Jeong J, Toida T, Muneta Y, Kosiishi I, Imanari T, Linhardt RJ, Choi HS, Wu SJ, Kim YS. Localization and Characterization of Acharan Sulfate in the Body of the Giant African Snail Achatina fulica: Comparative Biochemistry and Physiology. Biochemistry and Molecular Biology. 2001; 130(4): 513-9.
6. Vieira TCRG, Costa-Filho A, Salgado NC, Allodi S, Valente AP, Nasciutti LE, Silva LCF. Acharan Sulfate, the New Glycosaminoglycan from Achatina fulica Bowdich 1822. European Journal of Biochemistry. 2004; 271(4): 845-54.
7. Dominic J, Gem J, Frances M, Asis D, Guzman D, Mae J, Gracielle C, Monica MA. Formulation and Evaluation of Giant African Snail (Achatina fulica) Slime and Chitosan Composite Films for Cicatrization. Thesis: University of San Agustin; 2015: 20p.
8. Kementerian Kesehatan Republik Indonesia. Peraturan Menteri Kesehatan Republik Indonesia Nomor 6 Tahun 2016 Tentang Formularium Obat Herbal Asli Indonesia. Jakarta: Kementerian Kesehatan Republik Indonesia; 2016.
9. Rahmawati F, Widiyanti P, Ady J. Efek Penambahan Glycosaminoglycan dari Lendir Bekicot (Achatina fulica) pada Paduan Alginat – Carboxymethyl Cellulose (CMC) sebagai Accelerator Wound Healing. Jurnal Fisika dan Terapannya. 2015; 3(2): 93-104.
10. Suartiningsih A. Formulasi Sediaan Gel Lendir Bekicot (Achatina fulica) dengan Natrium Carboxymethyl Cellulose sebagai Gelling Agent untuk Penyembuhan Luka Bakar pada Kelinci Jantan. Thesis: Universitas Muhamadiyah Surakarta; 2011.
11. Santana WA, Ribeiro MAG, Cardoso JC, Melo CMde, Albuquerque-Junior RLCde. Effect of Combined Application of Dressing Films Based on Mucous Secretion of Achatina fulica and Low Level Laser Therapy on Wound. American International Journal of Contemporary Research. 2014; 4(7): 17-27.
12. Harti AS, Sulisetyawati SD, Murharyati A, Oktariani M, Wijayanti IB. The Effectiveness of Snail Slime and Chitosan in Wound Healing. International Journal of Pharma Medicini and Biological Sciences. 2016; 5(1): 76-80.
13. Alves RR, Rosa IL. Why Study the Use of Animal Products in Traditional Medicines?. Journal of Ethnobiology and Ethnomedicine. 2005; 1(5): 1-5.
14. Caetano GF, Fronza M, Leite MN, Gomes A, Frade MAC. Comparison of Collagen Content in Skin Wounds Evaluated by Biochemical Assay and by Computer-aided Histomorphometric Analysis. Pharmaceutical Biology. 2016; 54(11): 2555-9.
15. Novaes RD, Cupertino MC, Sarandy MM, Souza A, Soares EA, Gonçalves RV. Time-Dependent Resolution of Collagen Deposition During Skin Repair in Rats: A Correlative Morphological and Biochemical Study. Microscopy and Microanalysis. 2015; 21(06): 1482-90.
16. Santana WA, Melo CMde, Cardoso JC, Pereira-Filho RN, Rabelo AS, Reis FP, Albuquerque-Júnior RLCde. Assessment of Antimicrobial Activity and Healing Potential of Mucous Secretion of Achatina fulica. International Journal of Morphology. 2012; 30(2): 365-73.
17. Schultz GS, Chin GA, Moldawer L, Diegelmann RF. Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists. Adelaide: Barr Smith Press; 2011. 423-50p.
18. Orsted HL, Keast DH, Forest-Lalande L, Kuhnke JL, O'Sullivan-Drombolis D, Jin S, Haley J, Evans R. Skin: Anatomy, Physiology and Wound Healing. Canadian Association of Wound Care. 2018:1-25.
19. Marques-Marinho FD, Vianna-Soares CD. Cellulose and Its Derivatives Use in the Pharmaceutical Compounding Practice. 2013. http://dx.doi.org/10.5772/56637.
20. Gunawan, Hasan CY, Rahardjo. Pengaruh Perbedaan Konsentrasi Gel Lendir Bekicot Terhadap Ketebalan Epitel dan Luas Luka Pada Proses Penyembuhan Pascxa Eksisi Kulit. Thesis: Universitas Gadjah Mada; 2020: 59p.
21. Olczyk P, Menener A, Vassev KK. Review Article Diverse Roles of Heparan Sulfate and Heparin in wound Repair. Biomed Research International. 2015:1-5.