

ANGIOGENIC PROPERTY OF COLLAGEN FROM THE EGGSHELL MEMBRANE OF CHICKEN USING EX-OVO CHORIO-ALLANTOIC MEMBRANE ASSAY OF DUCK EGGS

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ABSTRACT

Ischemic heart disease remains one of the leading cause of death globally, and drug therapies, medical procedures and extracellular matrices such as heparin is used to promote formation of new blood vessels as the intervention for the disease. Angiogenesis is the process wherein new blood vessels form and grow on a pre-existing vasculature, wherein it is effective in treating or preventing ischemic heart disease. Collagen is abundant in the matrix of extracellular tissues of humans and plays a vital role in giving structural support and function on the growth and regeneration of tissues, which can be helpful in tissue engineering as its properties include high mechanical strength, low antigenicity, good biocompatibility and ability of being cross-linked that makes it an ideal biomaterial for such application. The purpose of this study is to utilize eggshell membrane, an industrial waste, to extract collagen and to determine its angiogenic property compared to standard drug, Heparin. Collagen was extracted from the separated membranes using acetic acid relative to membrane weight at a ratio of 1:4. The eggshell membrane collagen yield of 14.48%. At electron microscopic level, a distinct structure was observed having a spontaneously formed macro porous scaffolds similar to type 1 collagen. Fourier Transform Infrared Spectroscopy showed regions of amide A (3437.15), amide B (2929.87), amide I (1656.85), amide II (1558.48) and amide III (1247.94), which are essential functional groups for proteins and triple helical structure. Confirmation of purity was established by using High Performance Liquid Chromatography retention time comparison with the extracted collagen (1.803) and standard collagen (1.829). Analysis of Differential Scanning Calorimetry revealed that the thermal denaturation was about 106.85°C indicating high stability of structures. Chorio-allantoic membrane filter paper assay quantification was determined based on collateral counts. Concentration of 3mg/mL (p=0.169) and 10mg/mL(p=0.588) extracted collagen have no significant differences with the positive control, Heparin. At concentration of 5mg/mL (p=0.006) eggshell membrane collagen exhibited the most angiogenic activity greater than the positive control, Heparin. The results suggest that collagen of eggshell membrane could improve tissue perfusion reducing the occurrence of ischemic tissues.

KEYWORDS: angiogenesis, egg shell, collagen, chorio-allantoic membrane, extracellular matrix (ECM), growth factor (GF).

INTRODUCTION

Ischemic heart disease remains one of the leading cause of death globally in the last 18 years with 31% of all global deaths (WHO, 2016). Narrowed arteries on the heart cause ischemic heart disease, in which less oxygen and blood passes through the arteries (decreased levels of oxygen and blood on the heart is also known as cardiac ischemia), making it hard to reach the muscles of the heart that can lead to a heart attack. In present time, couple of medical procedures, therapies and medicines are used for preventing ischemic heart disease; such as taking HMG CoA reductase inhibitors, undergoing PCI procedure and CABG procedure.

Angiogenesis is the process wherein new blood vessels form and grow on a pre-existing vasculature. Therapeutic angiogenesis is said to be effective in treating or preventing ischemic heart disease and osteoarthritis; also, it can be used as skin filler, wound dressing and vascular prosthetics by restoring the circulation of blood in the arteries by promoting blood vessel formation to restore the circulation of blood in the arteries (Zhang et al., 2013).

The growth of blood vessels is maintained on the extracellular matrix (ECM) with the help of endogenous ECM proteins directly binding to the VEGF such as

collagen, while the commercially available Heparin also induces proliferation of the blood vessels and has a mechanism directly to the Heparin binding-sequence. There are many growth factors that help increase proliferation of blood vessels, but all of them exhibit different mechanisms.

Collagen is abundant in the matrix of extracellular tissues of humans and plays a vital role in giving structural support and functions on the growth of tissues and regeneration (Garigapati, 2013). Collagen can be acquired from many natural sources such as fruits, mammals, and amphibians. Apart from that, collagen is also abundant on the eggshells of a chicken egg (Woo *et al.*, 2008).

Eggshell is an abundant industrial waste that can be collected aplenty anywhere. It is composed of collagenous protein fibers that can be seen on the outer and inner membrane of the eggshell. Its membrane has a high percentage yield of amino acids such as hydroxyproline that is present in type I collagen (Wong *et al.*, 2008). This study determines the characteristics and angiogenic activity of the collagen from the eggshell membrane using CAM assay.

MATERIALS AND METHODS

1. Collagen Extraction from Eggshell Membrane

1.1 Collection of the Sample

The eggshells of chicken were collected at Rizal Poultry & Livestock Association, Inc. The eggshells were cleansed properly using distilled water.

1.2 Preparation, Separation, Pre-treatment, Extraction of Collagen from Eggshell Membrane

1.3 Separation of Eggshell Membrane: The eggshells were mixed with 5 liters of 5% EDTA solution and stirred at the speed of 50 rpm for 30 minutes, Heavier eggshell precipitated while the lighter membrane floated and collected. The eggshell membrane were then washed with distilled water.

1.4 Pre-treatment Process: The collected eggshell membrane were washed with cold distilled water using a blender for 3 minutes after that it was filtered, this was done to ensure that the sample is completely washed from the EDTA solution that was used. The membrane was mixed with 0.45M NaCl in 500 mL using a magnetic stirrer for 3 minutes. The retentate were then homogenized with another 0.45M NaCl in 500 mL at 6,000 rpm for 4 minutes, to assure that the membrane become neutral by gradually adding neutral salt solution. The membrane was then washed with distilled water and centrifuge at 2,000 g, 40C, for 30 minutes. The precipitate were collected and stirred with 0.2% NaOH, 0.2% H₂SO₄, and 0.7% citric acid, respectively, for 4 minutes to remove non-collagenous protein and cause a significant amount of swelling, It also provided the proper pH condition for extraction. The membrane were washed with distilled water to pH 7 and then filtered.

The retentate membrane were soaked with 10% NaCl for 24 hours at room temperature after that it was filter. The sample were bleached using 1% alkaline hydrogen peroxide in 0.01 M NaOH for 24 hours at room temperature for the sample to be neutralize. The eggshell membrane were finally washed with distilled water.

1.5 Extraction of Collagen: Collagen was extracted from membranes, obtained from the pre-treatment process, with 0.5 M acetic acid at a ratio of 1:4. Membranes were mixed with 0.5 M acetic acid, in a shaker water bath, at 40C for 2 hours. Mixtures were then centrifuged for 4 min at 6,000 rpm, again mixed in the shaker water bath at 40C for 24 hours, homogenized for 2 min at 6,000 rpm, 40C, and finally centrifuged at 10,000g, 40C for 20 minutes.

2. Percentage Yield Computation of the Egg Collagen

$$\text{percentage yield} = \frac{\text{weight of collagen after extraction}}{\text{weight of eggshell membrane before extraction}} \times 100$$

3. Morphological Evaluation

Scanning Electron Microscopy coupled with Energy Dispersive X-ray (SEM/EDX) Spectroscopy was used in the morphological analysis of the egg collagen.

4. Physical and Chemical Evaluation

The collagen extract was subjected to organoleptic test, solubility test, biuret test, ninhydrin test, xanthoproteic test, Hopkin's cole test, Millon's test, test for -SH group.

5. Instrumental Evaluation

A High-Performance Liquid Chromatography (HPLC) system at the Centro Escolar University-Makati was used with a wavelength of 214 nanometers applying a flowrate of 0.35 milliliters per minute on an Agilent BioSEC 300 Å column (300 mm length × 4.6 mm i.d., 3.0 μm). An isocratic gradient with a mobile phase consisting of 150 mM phosphate buffer, pH= 7.0, was used for the chromatographic separations. The column temperature was maintained at room temperature. The auto-sampler syringe and the injection valve were successively washed with distilled water (70/30; v/v). The injected sample volume into the system was 20 μL. Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) analysis as conducted at the Research Center for Natural and Applied Sciences (RCNAS) of University of Santo Tomas.

6. Documentation

The chick embryos growth stages were documented on daily basis and photographed. All of the chick embryos were photographed, however due to the nature of risk involving bacterial and fungal growth, out of 24 trials only 18 were able to be photographed until the 10th day.

7. Chorio-Allantoic Membrane Assay

7.1 Using Native Duck (*Anas platyrhynchos*) as Medium

The balut eggs were cleaned using paper tissue with 70% ethyl alcohol to remove debris and dirt under the laminar flow hood.

7.2 Pre-Incubation Process

The temperature was adjusted to 37.5C and relative humidity to 50-62% using a temperature and humidity meter. The plastic container and its cover were sterilized in 70% EtOH by dipping in a bath of EtOH and leaved to dry in a laminar flow hood. The top and bottoms of the plastic container which will act as lids were placed under UV rays in a laminar flow hood. After the pre-incubation period, all of the contents of the eggs were transferred in the properly covered plastic container.

7.3 Incubation Process – Ex ovo cultures were returned to an incubator, kept at 37.5C and 50-62% humidity and incubated for an additional 4 days (until developmental day 9; 8 days of incubation).

7.4 Preparation and Application of Controls and Experimental Substances

a. On day 8 of incubation, under sterile conditions within a laminar flow hood, an autoclaved filter paper (1 mm in diameter) were punched by a hole puncher and individually used as a supporting material/ carrier for controls and experimental substances. After locating an area containing a fine vessel networks between large blood vessels, the disk were lifted individually with fine end forceps and implanted onto the CAM.

b. The filter paper disk was used to confine the controls and experimental substances. The 1 mm sterilized filter paper disks were pre-soaked with used substance individually and dried before implanting on the CAM. The purpose was to make sure the used substance were concentrated and contained only on the chosen area of interest. The 1 mm sterilized filter paper disks may contain the following:

- Positive control: 10mg/mL of Heparin
- Negative control: Plain Normal Saline Solution
- Blank: Untreated
- Filter paper disk alone
- Experimental substance:
 - 10mg/mL of Collagen extract with 0.9 % Normal Saline Solution
 - 5mg/mL of Collagen extract with 0.9 % Normal Saline Solution
 - 3mg/mL of Collagen extract with 0.9 % Normal Saline Solution

7.5 Post Incubation – Samples were incubated for another 48 hours.

7.6 Microscopic Analysis – After 48 hours, the samples were removed from the incubator and mounted on the stereomicroscope for evaluation.

7.7 Quantification of the Number of Collaterals – Quantification of angiogenesis was accomplished by observing the following parameters: The blood vessels counted were near and beneath the filter paper disc applied. Only blood vessels that branched out near and beneath the confined region of the filter paper disc were tallied. Pre-existing large-vessels were not counted. Only newly formed thin branches were counted.

8. Statistical Treatment of Data

One Way ANOVA was utilized in determining the significant difference between the treatments. Post Hoc Analysis was employed to compare the significant difference between the experimental treatments.

RESULTS AND DISCUSSION

1. **Percentage Yield of Extracted Collagen:** The amount of eggshell membrane has a significant effect on percentage yield of separated membrane. The 51.8g of eggshell weight gave a 14.48% yield shown in Table1.

Table 1: Yield of Eggshell Membrane.

| | |
|---|----------|
| Weight of Collagen after Extraction | 7.5018g |
| Weight of Eggshell Membrane before Extraction | 51.8006g |
| Total Weigh of Extracted Collagen Obtained | 14.48% |

2. **Morphological Evaluation:** Figure 1 shows a scanning electron microscopic images of both collagen. Similar to Type 1 Rat tail collagen, the eggshell membrane collagen has a spontaneously formed macro porous triple helix scaffolds. Both collagen scaffolds of Rat tail and Extracted collagen have macro porous frameworks with high proportions of pore with the diameters ranging from 30 to 50 μm and 50 to 100 μm , respectively.

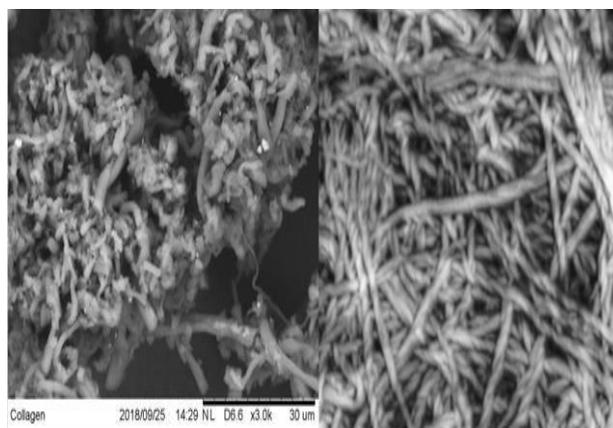


Figure. 1: Scanning Electron Microscopic images of Extracted Collagen (left image) and Collagen Type 1 (right image).

3. Chemical Evaluation

Table 2: Chemical Tests for proteins.

| Chemical Test | Expected Result | Actual Result | Description | Interpretation |
|---------------------|-----------------------------|---------------------------|---------------------------------|--|
| Biuret Test | Violet pink junction | Violet pink junction | General test for Proteins | Presence of Peptide Linkage |
| Ninhydrin Test | Blue Violet Solution | Blue Violet Solution | Presence of Alpha Amino Acid | Amino group attached to Alpha carbon |
| Xantho proteic Test | Yellow orange solution | Yellow orange precipitate | Presence of Aromatic Group | Aromatic residues accelerate the self-association process |
| Hopkins Cole Test | Red to Violet ring solution | Clear solution | Absence of Tryptophan | Tryptophan, Tyrosine and Cysteine are rare within the triple helix structures. |
| Millon's Test | Flesh to red solution | Brown solution | Absence of Phenol Group | |
| -SH group | Black Precipitate | Black Solution | Absence of -SH group (Cysteine) | |

4. Instrumental Evaluation

4.1 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy has been used to study changes in the secondary structure of collagen (Friess and Lee, 1996). The FTIR spectrum of eggshell membrane collagen is shown in Figure 2. The main absorption bands are amide A (3437.15), amide B (2929.87), amide I (1656.85), amide II (1558.48), amide III (1247.94). Amide A band is related to NH stretch coupled with hydrogen bond, amide B is related to CH₂ asymmetrical stretch. Amide II is associated with NH bending and CN stretching. Amide III is related to CN stretching and involved with the triple helical structure of collagen (Wang *et al.*, 2008; Woo *et al.*, 2008).

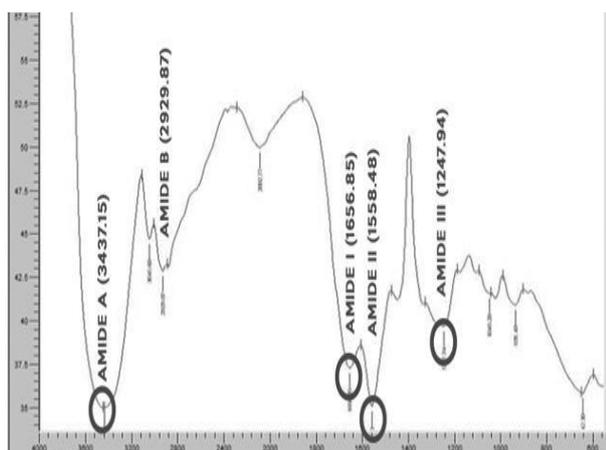


Figure 2: Fourier transform infrared spectrum of eggshell membrane collagen.

4.2 High Performance Liquid Chromatography

The confirmation of purity of the extracted collagen sample was established by using retention time comparison with the standard, Type 1 Collagen shown in Table 2.

Table 2: HPLC Retention time.

| | |
|----------------------------|-------|
| Eggshell Membrane Collagen | 1.803 |
| Type 1 Rat Tail Collagen | 1.829 |

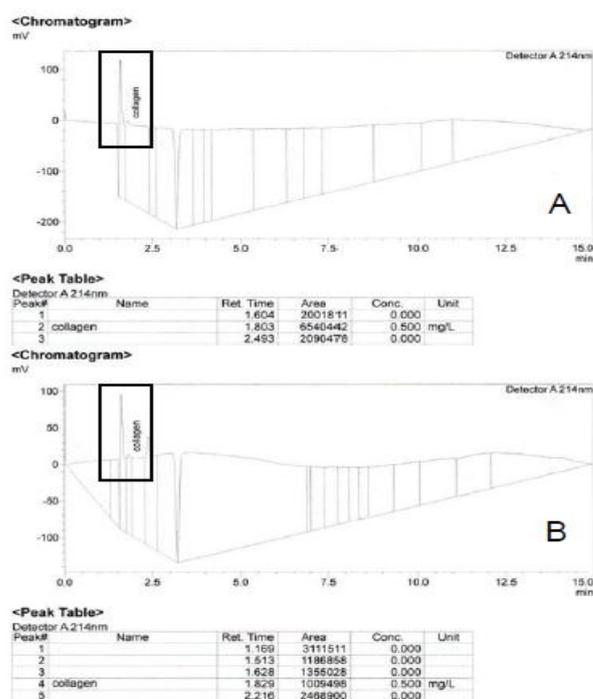


Figure 3: Chromatogram of Eggshell Membrane Collagen (A) and Type 1 Rat Tail Collagen (B).

4.3 Denaturation Temperature.

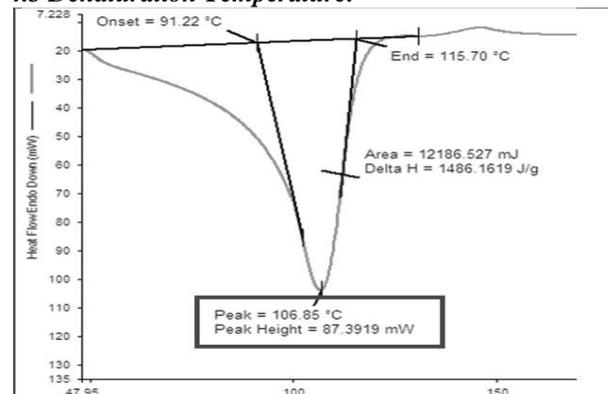


Figure 4: Differential Scanning Calorimetry Thermogram.

The Td of eggshell membrane collagen can be used as an effective index of assessing the stability of eggshell membrane collagen. With increase in temperature (thermal depolymerization process), the hydrogen bonds within collagen are broken progressively and finally, the triple helix structure of collagen maintained by hydrogen bonds is converted into random coil conformation of gelatin (Wang *et al.*, 2008). DSC thermogram of eggshell membrane collagen is shown in Figure 4. DSC thermogram showed the Td is determined to be about 106.85°C. The value of H of eggshell membrane collagen is 1486.1619 Jg-1.

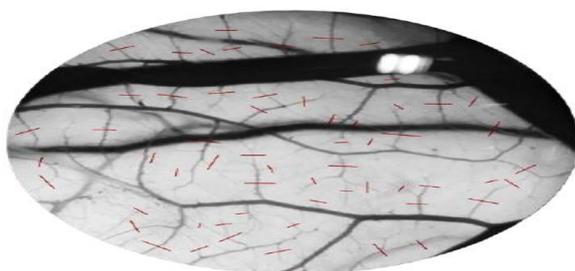
Td of eggshell membrane collagen is higher than pig skin collagen (60°C) and bovine skin (63-65°C) indicating that it thermally stable than other mammalian collagen.

5. Chorio-Allantoic Membrane Assay

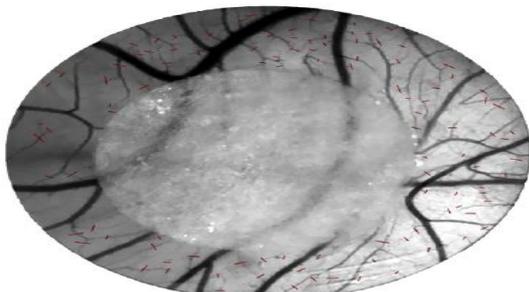
The results revealed that ANOVA Post Hoc Test p value of 0.015 (Untreated), 0.011 (Negative) and 0.027 (Positive) to the assigned level of significance at <0.05. Therefore the hypothesis that there is no significant difference to the average growth of blood vessels among the different doses of collagen extract is rejected. It means that there is a significant difference to the average growth of blood vessels among the different doses of collagen extract.

Figure 5 Chorio-allantoic membrane assay of *Anas platyrhynchos* subjected to experimental and control treatments. Break lines point to the secondary collaterals.. (a) Group 6: Untreated. (b) Group 4: Positive control, Heparin. (c) Group 5: Negative control, Normal Saline Solution.

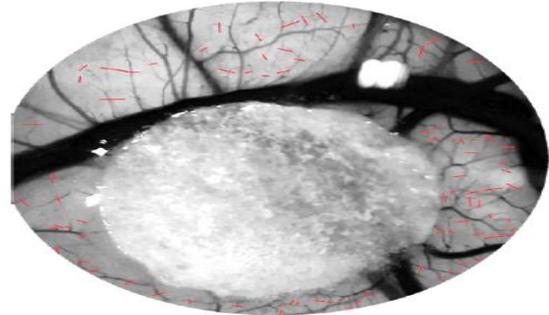
(d) Group 1: Collagen extract 3mg/mL. (e) Group 2: Collagen extract 5mg/mL. (f) Group 3: Collagen extract 10mg/mL.



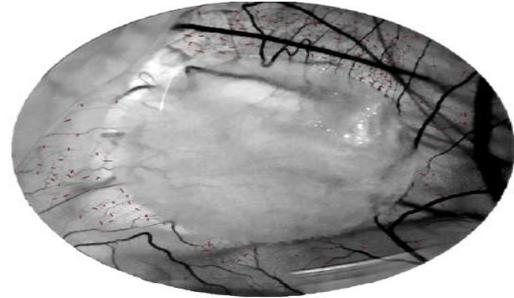
A



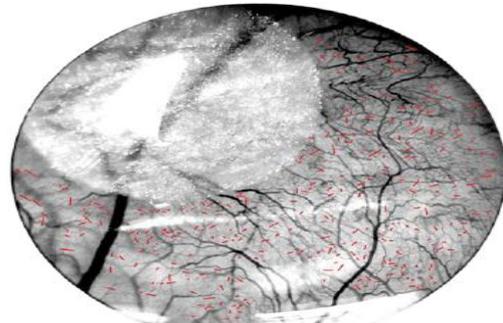
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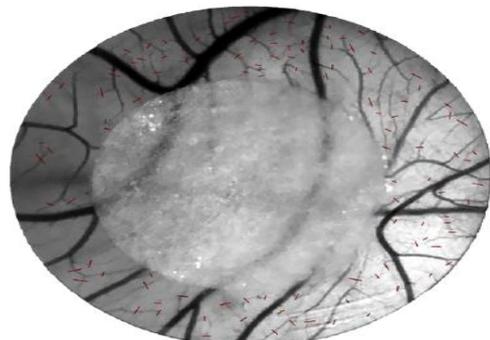
C



D



E



F

As shown in figure 5, It is found out that group 2 (5mg/mL collagen extract) had the highest average collateral count of 195.3, followed by the group 1 (3mg/mL collagen extract) with an average collateral count of 123.3. Group 3 (10mg/mL collagen extract) and Group 4 (positive control, Heparin) with the collateral counts of 91.6 and 73.3, respectively. Lastly, Group 6 (untreated) yielded and average collateral count of 58, while Group 5 (negative control, NSS) with an average

of 47.3. In comparison of Group 1 (3mg/mL collagen extract) and Group 2 (5mg/mL collagen extract) with the Group 5 (negative control, NSS) and Group 6 (untreated), the results showed significant differences to the groups, while the Group 3 (10mg/mL collagen extract) showed no significant difference with untreated and negative drug. The comparison among 6 groups have significant differences, especially on Group 2 (5mg/mL collagen extract) that has greater pro-angiogenic activity than Group 4 (positive control, Heparin).

CONCLUSION

The characteristics of type I collagen extracted from eggshell membrane were determined and proven to be comparable with standard collagen, type I rat tail collagen. There is a significant difference in the angiogenic activity of eggshell membrane collagen among the three control groups. At concentration of 5mg/mL, Eggshell membrane collagen exhibited the most angiogenic activity greater than the positive control, Heparin.

RECOMMENDATIONS

To further improve the presented results of this study, the researchers recommend the following:

1. To perform the use of ultrasound which proved to increase the yield and reduce the extraction time without damaging the quality of collagen.
2. To further use nylon mesh and histogram equalization of green channel image for easier quantification of the collateral vessels.
3. To add more instrumental test to determine the specific type of collagen such as Sodium Dodecyl Sulphate Polyacramide Gel Electrophoresis.
4. To find different source of such as duck eggs, pig intestine, quail eggs or any other biological waste with the presence of collagen that will provide a higher yield.
5. To make a formulation of the eggshell membrane collagen extract to reduce occurrence of ischemic tissue and for regenerative medicine.
6. To conduct a toxicity test on different doses to be administer to the eggs.

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