



# Collagen Extracted from Persian Gulf Squid Exhibits Anti-Cytotoxic Properties on Apple Pectic Treated Cells: Assessment in an In Vitro Bioassay Model

*Ladan DELPHI<sup>1</sup>, \*Houri SEPEHRI<sup>1</sup>, Elaheh MOTEVASELI<sup>2</sup>, Mohammad Reza KHORRAMIZADEH<sup>3</sup>*

1. Dept. of Animal Biology, School of Biology, College of Science, University of Tebran, Tebran, Iran

2. Dept. of Medical Genetics, Tebran University of Medical Sciences, Tebran, Iran

3. Endocrinology and Metabolic Research Institute, Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tebran University of Medical Sciences, Tebran, Iran

\*Corresponding Author: Email: hsepehri@khayam.ut.ac.ir

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## Abstract

**Background:** Collagen-based three-dimensional (3D) in vitro systems have been introduced to study the physiological states of cells. As a biomolecule, collagen is usually extracted from terrestrial animals whilst aquatic animals like squid contain large amounts of collagen.

**Methods:** In order to make effective use of marine organisms, we selected Persian Gulf squid in 2015 to extract the required collagen. Then, a 3D culture system based on the extracted collagen was applied to investigate cellular mechanisms in a native microenvironment. The formed collagen gel was used to investigate the growth of MDA-MB-231 breast cancer cells as well as responses to pectic acid.

**Results:** The results revealed that the extracted collagen contained  $\alpha$ ,  $\beta$  and  $\gamma$  components with high water holding capacity. This collagen formed a gel-like structure, which could promote the proliferation of MDA-MB-231 breast cancer cells. The MDA-MB-231 cells' viability in presence of pectic acid, demonstrating the cells' behavior in a 3D culture system.

**Conclusion:** It seems that the collagen extracted from squid skin has type I collagen properties. It might be used as a substrate in 3D cell culture systems.

**Keywords:** Cell culture, Collagen, Pectic acid, Squid, 3D culture system

## Introduction

Three-dimensional (3D) culture systems have recently gained increasing recognition as an effective tool for biological and biomedical research. Compared to conventional monolayer culture systems, these systems show higher resemblance with physiologic environment of living organisms (1). They have closer resemblance to the cells growing in the in vivo tissue environment (2). They further contain specific characteristics similar to the corresponding tissues in vivo and can

stay active and functional for weeks (3). It is important to study the cells' growth, proliferation and response to chemical agents in an environment organized with cell to cell and cell to matrix interactions plus a well-defined morphological geometry. This would be approached with 3D in vitro systems (4).

Monolayer cell culture systems of cancer cell lines are commonly used to evaluate the antitumor effects of anticancer drugs (5). Most tumor cells

are supported by an extracellular matrix microenvironment, with an important role in resistance against anticancer drugs (6). Three-dimensional culture systems have allowed the study of cell-to-cell interactions and tumor-mediated angiogenesis (3). In addition, spheroid cultures that use a compound derived from basement membrane, organotypic cultures, and soft agar cultures as well as cells embedded in collagen gel have been developed to investigate the biology of cancer cell lines (7). We have used the collagen extracted from Persian Gulf squid (*Uroteuthis duvauceli*) as a 3D culture system to study the capacity of pectic acid to inhibit tumor cells' growth in MDA-MB-231 breast cancer cells.

Whole apple extract can inhibit mammary cancer in a dose-dependent manner in rat model (8). The major component of apple is a kind of polysaccharide called pectin. Dietary pectic and its degradation products (pectic oligosaccharides) caused decreased proliferation in HT-29 cells (9). There are a number of studies related to pectic (mostly citrus pectin) and cancer. Studies on the effect of pectic on different types of cancers such as blood-borne, prostate and colorectal indicated that pectic intake was beneficial to inhibit tumor growth and metastasis (10, 11). In addition, the effect of apple pectic on colon cancer through enhancement of apoptosis and inhibition of tumor formation has been reported both on animal and cellular models (12, 13).

The main objective of this research was to apply the collagen extracted from Persian Gulf squid as a biomaterial to be used in cell culture systems in order to study the development of anticancer drugs and treatments.

## **Methods**

### ***Collagen extraction***

Squids were harvested from the Persian Gulf water in 2015. Collagen was removed from the squid skin (14). The skin was mechanically separated from the squid body, defatted in 10% butyl alcohol, and rinsed in acetic acid and NaCl. Finally, the collagen precipitates were dissolved in 0.5 M

acetic acid and lyophilized. Collagen yield was measured based on the weight of skin. The total collagen obtained was determined according to AOAC using standard procedures (15). Analyses were conducted according to the weight of dry skin.

### ***SDS-Polyacryl Amide Gel Electrophoresis (SDS-PAGE)***

SSD-PAGE analysis was performed on 12% resolving gel and 5% stacking gel (16). The crude extracted collagen was dissolved in phosphate buffer containing 1% SDS and 0.5 M urea (Merck) (17) to give a final concentration of 5mg/ml. The gels were stained with Coomassie Brilliant Blue R-250 (Merck) and detained in methanol/acetic acid. Finally, the gels were captured.

### ***Cell culture***

To characterize the extracted collagen, MDA-MB-231 breast cancer cell line was grown on the squid collagen. The MDA-MB-231 cell line was obtained from NCBI (National Cell Bank of Iran). The cells were grown in RPMI 1640 medium (Gibco) in tissue culture flasks at 37 °C in 5% CO<sub>2</sub> until reaching confluence. The medium contained 10% Fetal Calf Serum (FCS) (Gibco) and 0.1% penicillin-streptomycin. The medium was changed two times a week. The cells were detached with 0.25% (w/v) trypsin-EDTA (Sigma). Cell number and viability was determined using trypan blue (Sigma) exclusive dye and hemocytometer.

### ***Collagen gelation***

The extracted collagen was prepared at 5 mg/ml concentration by suspending in sterile 0.01M acetic acid and stirring overnight at 4 °C. The gel was prepared on ice by adding DMEM/Ham' F12 3X, 5% FBS and NaOH to neutralize its acidity. To form the gel, collagen solutions were put in a CO<sub>2</sub> incubator at least for 30 min. Then, MDA-MB-231 cells were decanted onto the collagen in two models: collagen-coated and collagen gel (collagel). For both models, MDA-MB-

231 cells were treated with different concentrations of pectic acid (0, 0.5, 1.5, 3 mg/ml) for 24 and 48 h.

### MTT assay

The effect of pectic acid on cell proliferation was measured using MTT-based assay. The cells were plated on 96-well plates ( $1.0 \times 10^4$  cells). Then, they were treated with pectic acid. The cells were incubated with  $10\mu\text{l}$  of 5mg/ml MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazoliumbromide) solution for 3 h in phosphate-buffered saline (PBS). The formazan crystals produced from MTT were dissolved in DMSO, and their absorbance was recorded by ELISA reader at 630nm. The proliferation rate (PR) was calculated based on the following formula:

$$\text{PR percentage} = \frac{[\text{absorbance of drug treatment group} - \text{absorbance of blank}]}{[\text{absorbance of control group} - \text{absorbance of blank}]} \times 100.$$

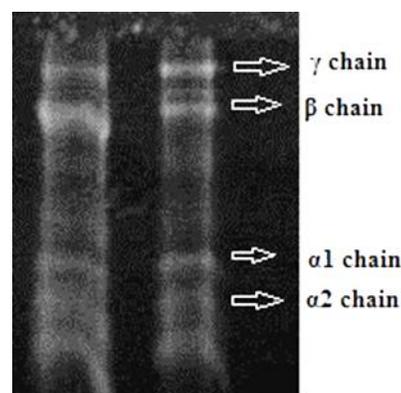
### Statistical analysis

All the experiments were done at least 3 times, and the data were shown as mean  $\pm$  standard error of the mean (SEM). The results were analyzed for significance using ANOVA test with Tukey's post hoc test.  $P \leq 0.05$  was considered significant.

## Results

### Physicochemical properties of squid collagen

The collagen content of the outer sheath of squid skin is high (18). The skin collagen content of Persian Gulf squid was about 3%, the extracted collagen contained low amounts of fat (0.5%) and approximately 98% moisture (Table 1). In SDS-PAGE pattern, the extracted collagen contained two  $\alpha$  chains, one  $\beta$  chain and  $\gamma$  chain components ( $\alpha 1$  about 50 KDa and  $\alpha 2$  about 80 KDa); accordingly, it was similar to type I collagen (Fig. 1).



**Fig. 1:** SDS-PAGE patterns of squid collagen showing that the collagen extracted from squid contained  $\alpha$  and  $\beta$  chains

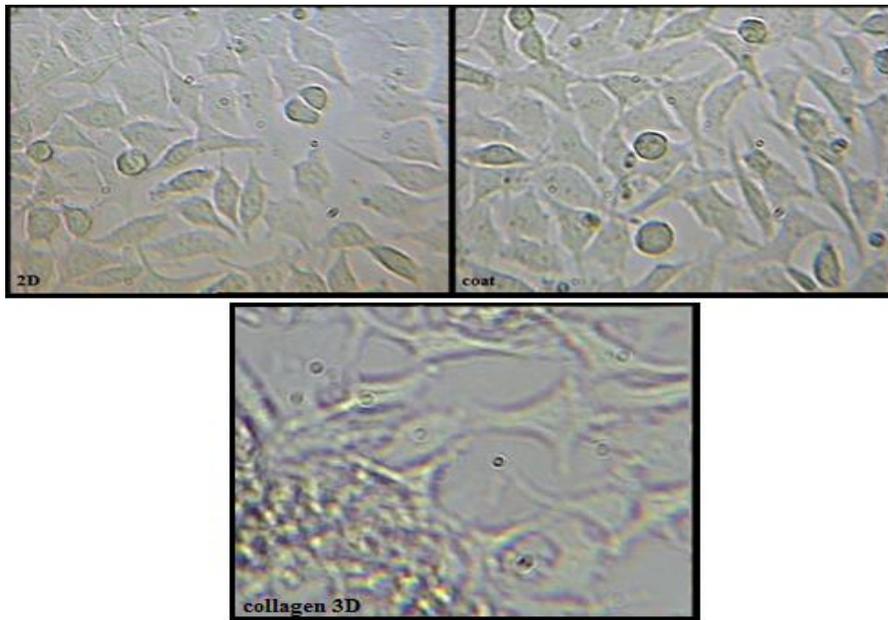
**Table 1:** Physicochemical properties of collagen extracted from Persian Gulf squid

Sample	Moisture (%)	Crude protein (%)	Collagen content (%)
Body skin collagen	98.3	0.9	2.61
Tentacle skin collagen	97.1	0.86	2.34

### Cell morphology in collagen cultures

MDA-MB-231 cells displayed flat cell bodies and a sheet-like monolayer spread over the culture plates in 2D in vitro system. To investigate the extracted collagen as a scaffold for cell culture, MDA-MB-231 cells were cultured in squid collagen gels for a period of four days. Cell-matrix interactions were seen as elongated morphology

with disorganized nuclei. Additionally, the cells began to proliferate and show cell-to-cell interactions, especially in collagen-coated cultures (Fig. 2). These interactions (cell to cell and cell to matrix) closely resembled in vivo biological systems. 3D cell populations were detected in the collagen extracted from squid.

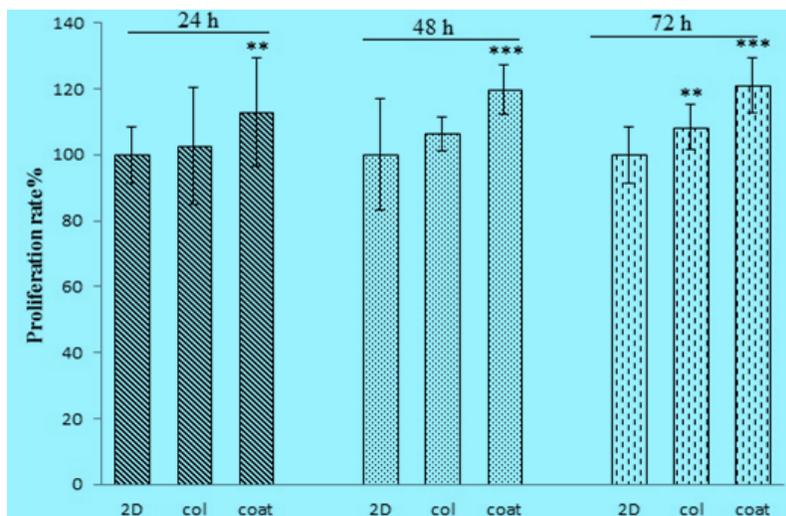


**Fig. 2:** MDA-MB-231 cells 48 h after cell cultivation on collagen extracted from Persian Gulf squid. 3D population of cells can be seen in the collagen cultures (100X)

### Cell proliferation in collagen cultures

In order to peruse the proliferation of MDA-MB-231 cells on collagen, the cells were cultivated on the squid collagen, and their proliferation rate was assessed for a period of 72 h. MTT test showed that the proliferation rate of cells was increased in collagen-coated plates after 24 h (Fig. 3). MDA-MB-231 cells cultivated on the

squid collagen did not show the same increase in proliferation rate as did the coated cells. The proliferation rate of the cells was significantly increased after 48 h in both the coated and squid collagen cultures, and the cells' density was so high in 96 h tests (Fig. 3). Therefore, squid collagen could promote cell proliferation when used as a culture matrix.

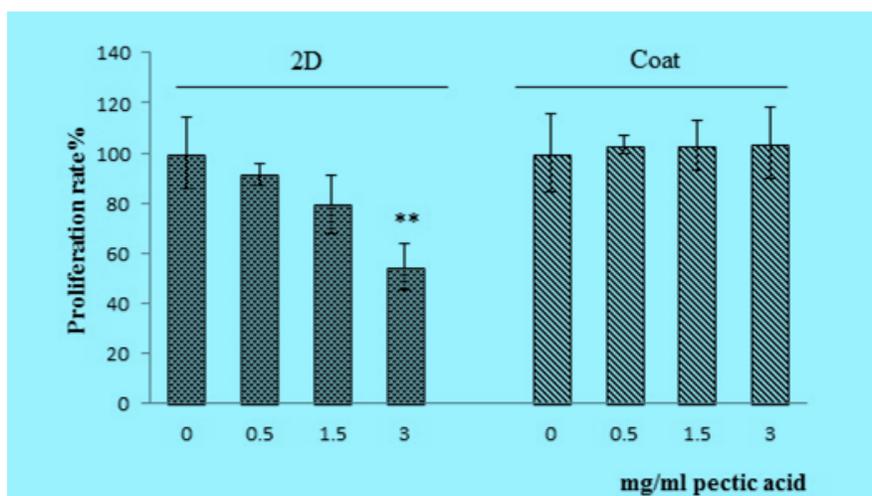


**Fig. 3:** MDA-MB-231 cells cultured in the collagen extracted from squid. As shown, proliferation rate is higher in the cultures containing collagen. Each value shown is mean $\pm$ SD (n=8). \*Significantly different from cells grown in 2D condition (\*\* $P$ <0.01, \*\*\* $P$ <0.001)

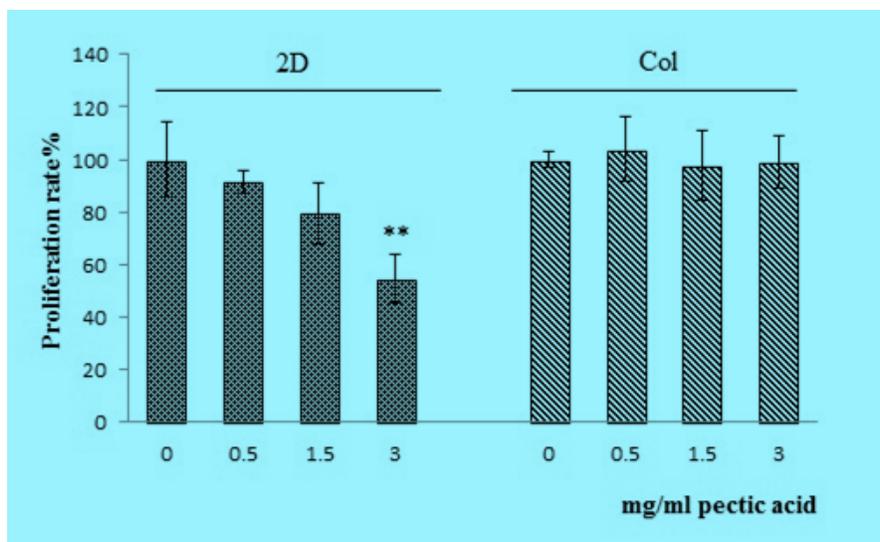
**Effect of pectic acid on the MDA-MD-132 cells in the collagen cultures**

In the first step, cell proliferation assay was performed to determine the effect of pectic acid on MDA-MB-231 cells. To reach this goal, MDA-MB-231 cells were treated with different concentrations of pectic acid (0, 0.5, 1.5, 3 mg/ml) for 24 h. Fig. 4 indicate that pectic acid inhibited cell growth and reduced the number of attached cells after 24 h. In this condition, degenerative

changes like loss of cell sheet were detected. Thus, pectic acid had a cytotoxic effect on MDA-MB-231 cells in 2D in vitro system. Subsequently, the culture of MDA-MB-231 cells on the squid collagen was treated with pectic acid. Even higher concentration of pectic acid (3 mg/ml) could not inhibit the MDA-MB-231 cells' growth both on collagen-coated and collagel cultures (Fig. 4 and Fig. 5).



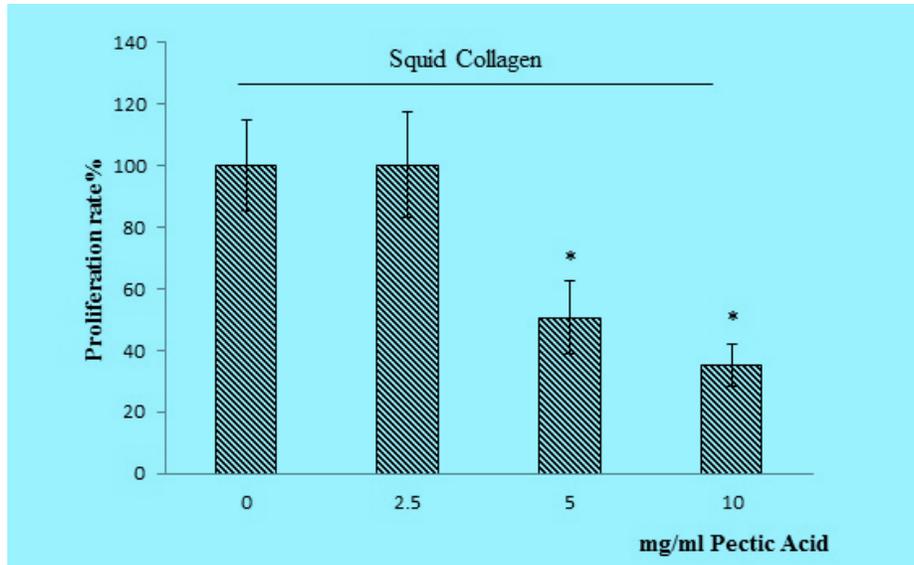
**Fig. 4:** Treatment with pectic acid for 24 h in 2D conventional condition inhibited the growth of MDA-MD-231 cells while pectic acid could not affect the cells in collagen-coated cultures. Columns show percent viable cells (n=8); \*\* indicate significant differences ( $P<0.01$ )



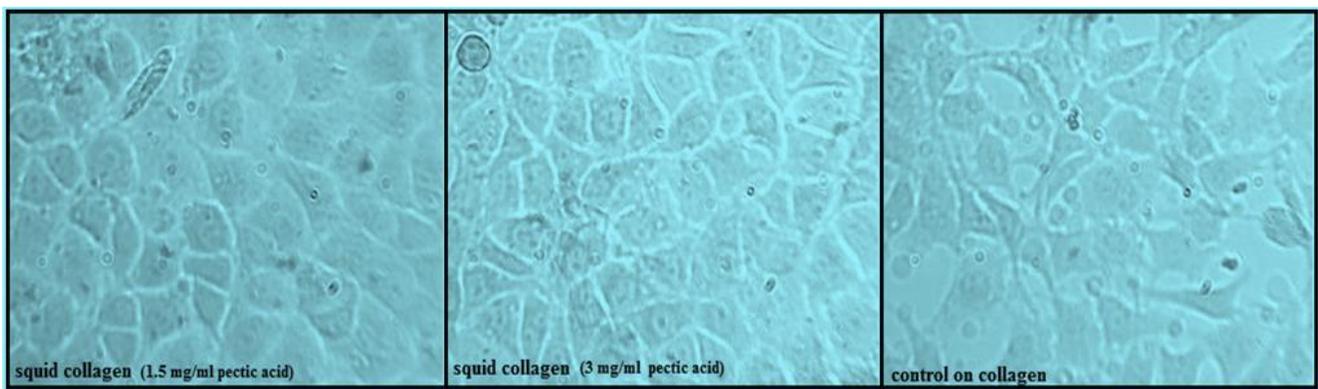
**Fig. 5:** Treatment with pectic acid for 24 h on the collagel culture obtained from the squid indicated no significant effect. Columns show percent viable cells (n=8); \*\* indicates significant differences ( $P<0.01$ )

To verify this result, higher concentrations of pectic acid (5 and 10 mg/ml) were applied on the cells. In these circumstances, pectic acid could show cytotoxic effect on MDA-MB-231 cells (Fig. 6). Morphological analysis of the cells indicated no obvious changes in culture of MDA-MB-231 cells on the squid collagen (coated and collagel) in presence of pectic acid (Fig. 7); how-

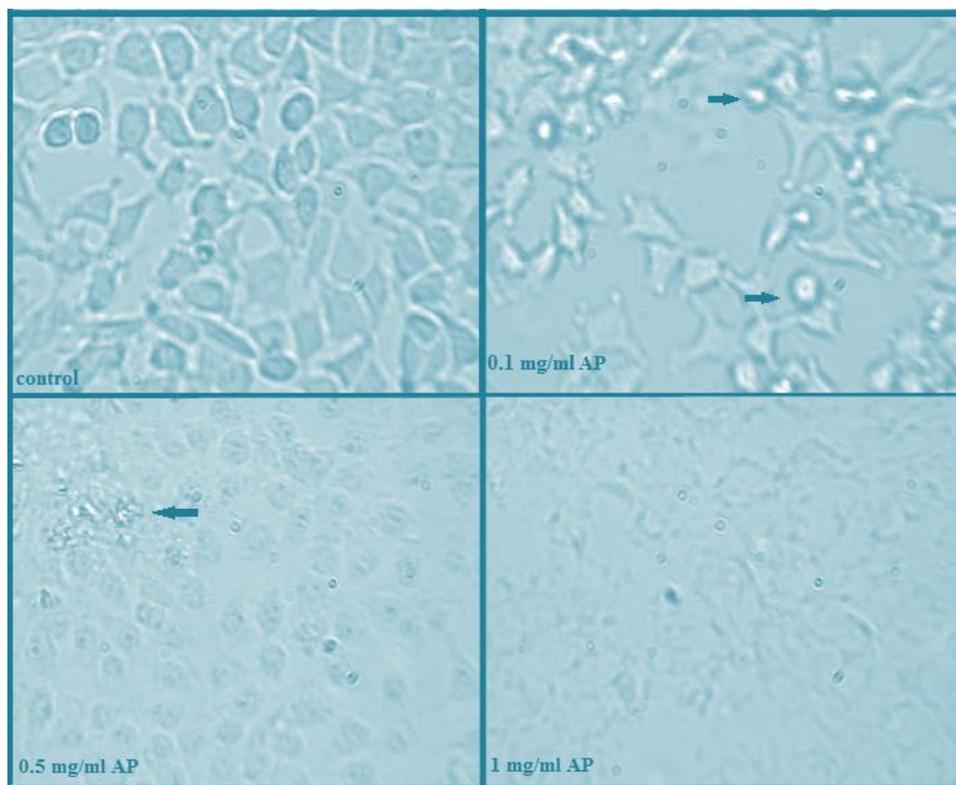
ever, the cells treated with pectic acid in 2D cultures showed changes such as irregular cell walls and cell debris in the medium. The majority of cells were floating, became rounded, and detached from the surface (Fig. 8). Therefore, the collagen extracted from squid could mimic 3D culture conditions through increased resistance of cells to chemical agents.



**Fig. 6:** Treatment with higher concentrations of pectic acid for 24h on the collagel culture inhibited the growth of MDA-MB-231 cells. Columns show percent viable cells (n=4); \*\* indicates significant differences ( $P<0.01$ )



**Fig. 7:** Morphological changes of MDA-MB- 231 cells treated with pectic acid on the collagen extracted from squid. There is no significant change in cell morphology compared to control cells (100X)



**Fig. 8:** Morphological changes of the MDA-MB- 231 cell treated with pectic acid on 2D conventional culture system. Cell debris and irregular rounded cell shapes are seen by increasing of pectic acid concentration (100X)

## Discussion

Collagen, a major component of all tissues, has various structural functions. This protein is regarded as one of the best candidates for several medical applications. Collagen shows excellent biocompatibility, safety and high biodegradability (19). Collagen is involved in many cellular processes, including regulation of cell motion, cell proliferation and apoptosis (20). For example, Type I collagen has been used as coating for culture dishes or as scaffold for microbiological adherence and invasion test systems. A 3D-culture matrix of collagen can act as a support for cells and mimic the real extracellular matrix (ECM) (21). Alternative sources of collagen have been developed since the use of main sources of collagen (land-based animals such as bovine or porcine) involves hygienic, social and cultural concerns. Marine creatures might offer a good

collagen source since high obtainability, lack of disease transmission risk and religious barriers as well as extraction capacity of high-yield collagen (22, 14). Moreover, fish waste, including bones, skins, scales as well as squid collagenous membranes detached during mechanical processing may be a new source for collagen (23). The collagen extracted from Persian Gulf squid (*Uroteuthis duvauceli*) contained Type I collagen, and could be formed as collagel. The extracted collagen could be used as a culture matrix for cell culture systems. Finally, the MDA-MB-231 breast cancer cells grown in 3-D culture of squid collagel are more resistant to pectic agents than the cells in 2-D culture.

The cells cultured within three-dimensional (3D) conditions exhibited phenotypes and responses to stimuli analogous to in vivo biological systems. This characteristic can be used in tissue engineering and in vitro tumor models (24). Morphological and protrusion analyses of MDA-MB-23 cells

indicated that cell to cell and cell to matrix interactions occurred during incubation of cells within the collagen extracted from Persian Gulf squid. In addition, the collagen extracted from Persian Gulf achieved biocompatibility to support cellular proliferation. These results were observed in both the collagen-coated and collagen cultures, which are consistent with a report (25). In this condition, the cell's response to drugs was significantly changed. The cells cultured in 3D culture systems; we expect to obtain an in vivo phenotype (26). Co-culture of MDA-MB-231 cells with squid collagen caused higher concentrations of pectic acid to inhibit proliferation of cells. Pectic acid could inhibit MDA-MB-231 breast cancer cell growth, correlated with cell death and apoptosis induction (27). The data achieved in this study indicated that the half-maximal inhibitory concentration of pectic acid was about 1 mg/ml when the MDA-MB-231 cells were cultured in a 2D traditional system. This concentration showed a 5-fold increase in the 3D culture system using the collagen extracted from Persian Gulf squid.

Squid collagen contains a porous structure sufficient to promote cellular adhesion, aggregation, morphogenesis and growth. As SEM analysis showed, a fibrous network and porous structure was observed including interconnected joints in an irregular and wavy shape for the extracted collagen (data not shown). These data imply that the collagen extracted from Persian Gulf squid is capable of generating a 3D culture system, which closely mimics in vivo conditions. Our data are in agreement with the report that showed 3D type I collagen system maintained the growth of MDA-MB-231 and MCF-7 breast cancer cells and decreased the cytotoxicity of apoptotic drugs such as Adriamycin in this system (28-29). In addition, the MDA-MB-231 cells cultured in 3D hydrogel of collagen scaffolds demonstrated greater resistance to paclitaxel as a cytotoxic chemotherapeutic agent (30). In all such these studies, collagen I extracted from rat-tail was used as cell 3D scaffold. The collagen extracted from Persian Gulf squid has functional properties like the rat-tail one. It may be applied in 3D cell culture systems.

## Conclusion

Conventional cell culture models are useful to investigate certain biological functions; however, physiologically relevant 3D cell culture models, including the one used in this study, have the potential to yield more effective and cost-efficient results for development of anticancer drugs and treatments.

## Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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## References

1. Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, et al. (2013). Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol*, 87(8):1315-530.
2. Ma HL, Jiang Q, Han S, Wu Y, Cui Tomshine J, Wang D, Gan Y, Zou G, Liang XJ (2012). Multicellular tumor spheroids as an in vivo-like tumor model for three-

- dimensional imaging of chemotherapeutic and nano material cellular penetration. *Mol Imaging*, 11(6): 487–498.
3. Kunz-Schughart LA, Kreutz M, Knuechel R (1998). Multicellular spheroids: a three-dimensional in vitro culture system to study tumour biology. *Int J Exp Pathol*, 79(1):1-23.
  4. Heppner GH, Miller BE (1989). Therapeutic implication of tumor heterogeneity. *Semin Oncol*, 16(2): 91-105.
  5. Howes AL, Richardson RD, Finlay D, Vuori K (2014). 3-dimensional culture systems for anti-cancer compound profiling and high-throughput screening reveal increases in EGFR inhibitor-mediated cytotoxicity compared to monolayer culture systems. *PLoS One*, 9(9): e108283.
  6. Corn PG (2012). The tumor microenvironment in prostate cancer: elucidating molecular pathways for therapy development. *Cancer Manag Res*, 4: 183-93.
  7. Breslin S, O'Driscoll L (2013). Three-dimensional cell culture: the missing link in drug discovery. *Drug Discov Today*, 18(5-6):240-9.
  8. Liu RH, Liu J, Chen B (2005). Apples prevent mammary tumors in rats. *J Agric Food Chem*, 53(6):2341-3.
  9. Olano-Martin E, Rimbach GH, Gibson GR, Rastall RA (2003). Pectic and pectic oligosaccharides induce apoptosis in in vitro human colonic adenocarcinoma cells. *Anticancer Res*, 23(1A): 341–6.
  10. Demotte N, Wieërs G, Van Der Smissen P, Moser M, Schmidt C, et al. (2010). A galectin3 legend corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer Res*, 70(19): 7476–7488.
  11. Morris V, Belshaw N, Waldron K, Maxwell E (2013). The bioactivity of modified pectic fragments. *Bioactive Carbohydrates and Dietary Fiber*, 1(1): 21–37.
  12. Li Y, Niu Y, Wu H, Sun Y, Li Q, Kong X, Liu L, Mei Q (2010). Modified apple polysaccharides could induce apoptosis in colorectal cancer cells. *J Food Sci*, 75(8):H224-9.
  13. Li Y, Liu L, Niu Y, Feng J, Sun Y, Kong X, Chen Y, Chen X, Gan H, Cao S, Mei Q (2012). Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitis-associated colon cancer: role of galectin-3 and apoptosis in cancer prevention. *Eur J Nutr*, 51(1):107–117.
  14. Senaratne LS, Park P, Kim S (2006). Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresour Technol*, 97(2): 191-197.
  15. AOAC. *Official methods of analysis*. 16<sup>th</sup> ed. Association of Official Analytical Chemists, Washington, DC, USA. 1995.
  16. Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259):680-5.
  17. Kolodziejska I, Sikorski ZE, Niecikowska C (1999). Parameters affecting the isolation of collagen from squid (*Illex argentinus*) skins. *Food Chem*, 66(2): 153–157.
  18. Jenkins CL, Bretscher LE, Guzei IA, Raines RT (2003). Effect of 3-hydroxyproline residues on collagen stability. *J Am Chem Soc*, 125 (21): 6422-6427.
  19. Lee C, Singla A, Lee Y (2001). Biomedical applications of collagen. *Int J Pharm*, 221(1-2):1-22.
  20. Cho SY, Klemke RL (2000). Extracellular-regulated kinase activation and Cas/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. *J Cell Biol*, 149(1): 223–236.
  21. Tibbitt MW, Anseth KS (2009). Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng*, 103(4): 655-63.
  22. Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, Tanaka, M (2005). Isolation and characterization of acid and pepsin-solubilised collagens from the skin of

- Brown stripe red snapper (*Lutjanus vitta*). *Food Chem*, 93: 475-484.
23. Yata M, Yoshida C, Fujisawa S, Mizuta S, Yoshinaka R (2001). Identification and characterization of molecular species of collagen in fish skin. *J Food Sci*, 66(2): 247-251.
  24. Szot CS, Buchanan CF, Freeman JW, Rylander MN (2011). 3D in vitro bioengineered tumors based on collagen I hydrogels. *Biomaterials*, 32(31): 7905-12.
  25. Kim JB (2005). Three-dimensional tissue culture models in cancer biology. *Semin Cancer Biol*, 15(5): 365-377.
  26. Hutmacher DW, Horch RE, Loessner D, Rizzi S, Sieh S, Reichert JC, et al. (2009). Translating tissue engineering technology platforms into cancer research. *J Cell Mol Med*, 13(8A): 1417-27.
  27. Delphi L, Sepehri H, Khorramizadeh MR, Mansoori F (2015). Pectic-Oligosaccharides from Apples Induce Apoptosis and Cell Cycle Arrest in MDA-MB-231 Cells, a Model of Human Breast Cancer. *Asian Pac J Cancer Prev*, 16(13):5265-5271.
  28. Koutsilieris M, Reyes-Moreno C, Choki I, Sourla A, Doillon C, Pavlidis N (1999). Chemotherapy cytotoxicity of human MCF-7 and MDA-MB 231 breast cancer cells is altered by osteoblast-derived growth factors. *Mol Med*, 5(2): 86-97.
  29. Krause S, Maffini M, Soto A, Sonnenschein C (2010). The microenvironment determines the breast cancer cells' phenotype: organization of MCF7 cells in 3D cultures. *BMC Cancer*, 10:263-270.
  30. Fang JY, Tan SJ, Yang Z, Tayag C, Han B (2014). Tumor bioengineering using a transglutaminase crosslinked hydrogel. *PLoS One*, 9(8): e105616.