

Effect of Dexamethasone on *In vitro* Maturation and Subsequent Fertilization of Buffalo Oocytes

¹A.S.S. Abdoon, ²S.A. Aziz, ¹O.M. Kandil, ¹A.A. Said and ²R.M. Sabrah

¹Department of Animal Reproduction and Artificial Insemination,
National Research Center, Giza, Egypt

²Department of Pharmacology, Faculty of Veterinary Medicine,
Zagazig University, Zagazig, Egypt

Abstract: This study was conducted to clarify the effect of the synthetic glucocorticoid dexamethasone (DEX) on the developmental competence of buffalo oocytes *in vitro*, two experiments were conducted. In experiment 1, buffalo cumulus-oocyte-complexes (COCs) were exposed during *in vitro* maturation (IVM) to 0.0 (control) or 1.0, 10, 50, 100 µg/ml of DEX. COCs were IVM in TCM-199 + 10% FCS, 10 µg/ml FSH, 50 µg/ml gentamicin. IVM was performed at 38.5°C for 24 h under 5%CO₂ in humidified air. After IVM, cumulus cells were removed by gentle pipetting, then fixed in 1:3 (v/v) Acetic acid: ethanol then stained with 1% aceto-orcein for detection of the nuclear configuration. The percentage of oocytes reaching the metaphase II (M II) stage (nuclear maturation) was recorded. In experiment 2, IVM was performed as in experiment 1 then the matured oocytes were *in vitro* fertilized (IVF) in presence of the same concentrations of DEX, *in vitro* matured buffalo oocytes were *in vitro* fertilized (IVF). After 18 h of oocyte-sperm co-incubation, the presence of first and second polar bodies as evidence of fertilization was detected under microscope. Results indicated that the percentage of buffalo oocytes reaching M II stage was significantly higher ($P<0.05$) in control than DEX treated groups. DEX even at the lowest concentration (1.0 µg/ml) caused oocyte activation (presence of two polar bodies and spontaneous cleavage). Fertilization rate was significantly higher ($P<0.05$) in control compared with the DEX treated groups in a dose dependent manner. Conclusion, based on these *in vitro* findings, DEX has an influence on buffalo oocyte maturation and subsequent fertilization rate.

Key words: Dexamethasone • Buffalo • Oocytes • Maturation Rate • Fertilization Rate

INTRODUCTION

Glucocorticoids (GCs) potentially affect normal gonadal function by acting at any one or more of the following levels in hypothalamic-pituitary-gonadal axis: 1) the hypothalamus (To decrease the synthesis and the release of GnRH); 2) the anterior pituitary gland (To inhibit the synthesis and release of gonadotropins); 3) the testis/ovary (To modulate steroidogenesis and/or gametogenesis directly. Concerning the effect of GCs on oogenesis, it has been shown that cortisol concentration in follicular fluid of pre-ovulatory follicles is higher than in plasma, suggesting a positive correlation between GCs and oocyte maturation [1].

Studies concerning the direct effect of glucocorticoids on oogenesis have shown conflicting results. *In vitro* maturation (IVM) of pig oocytes was found to be inhibited in a concentration- and time-dependent manner by glucocorticoids (Cortisol and dexamethasone) when added to culture media [2]. This inhibitory action of cortisol and dexamethasone on porcine oocytes was partially attributed to the reduction in p34^{cdc2}-cyclin B1 complex (maturation/metaphase-promoting factor, MPF) [3]. In addition, a follicle bioassay showed the toxic potential of dexamethasone on mouse ovarian function and early embryo development [4]. However, cortisol and dexamethasone did not affect meiosis resumption and maturation rate of mouse oocytes

undergoing FSH-induced and spontaneous IVM [5]. In women, oocytes that failed at fertilization came from follicles with higher cortisol levels in comparison to that yielded oocytes that were able to fertilize and cleave [6]. These discrepancies could be due, in part, to differences between species and strongly suggest that a single animal model may not be suitable to understand drug/chemical toxic actions on oocyte maturation and fertilization and their underlying molecular mechanisms.

The study of Gonzalez *et al.* [7] shows a differential effect of the natural glucocorticoid, corticosterone, and a synthetic one, dexamethasone, on mouse oocytes. Overall, a short exposure to high concentrations of corticosterone decreased *in vitro* oocyte maturation and, in mature oocytes pre-exposed to this glucocorticoid, it affected the ability to undergo fertilization and early *in vitro* development up to the blastocyst stage. Thus, both nuclear and cytoplasmic maturation may be hampered. However, dexamethasone had no effect on *in vitro* oocyte maturation, fertilization or cleavage rate. Dexamethasone had little effect, if any, on ERK-1/2 phosphorylation. Another study by Gonzalez *et al.* [8], using sheep oocytes as model system, shows for the first time an inhibitory effect of the glucocorticoids cortisol and dexamethasone on mitogen-activated protein kinases (MAPK) activation during *in vitro* oocyte maturation. Furthermore, this study shows an inconsistent effect of these glucocorticoids on oocyte progression through meiosis, with cortisol inhibiting and dexamethasone having no effect. None of these glucocorticoids affected the ability of oocytes that matured in their presence to undergo fertilization and early development.

In the literature, there are no reports for *in vitro* studies on bovine animal model. Therefore, this study was conducted to investigate the effect of direct exposure to dexamethasone on maturation rate of buffalo oocytes *in vitro*; and to clarify the direct effect of dexamethasone on buffalo the subsequent fertilizing capacity *in vitro*.

MATERIALS AND METHODS

All chemicals used in the present study are purchased from Sigma Company (Saint Louis, MO, USA), unless otherwise mentioned.

Experiment 1: Effect of Direct Exposure to Dexamethasone on *In vitro* Maturation Rate of Buffalo Oocytes

Chemical Exposure: Stock concentration of DEX was prepared in distilled water. Working concentrations were

prepared by serial dilutions in culture medium. DEX is added at the beginning of culture of the oocytes. The used concentrations were (0, 1, 10, 50, 100 µg/ml) similar to those used in earlier *in vitro* studies [2, 4, 5] and the range of DEX concentrations tested in this study included both physiological and supraphysiological levels of glucocorticoid (Cortisol) [9, 10]. DEX was used as it is a synthetic and potent steroid with a structure similar to that of cortisol but with higher potency [11] and it was used to enhance the potential effects of glucocorticoids.

Oocyte Collection and *in vitro* Maturation: Buffalo ovaries were collected at El Moneib slaughterhouse, transported to the laboratory within 1-2 hr. Upon arrival, the ovaries were washed once with 70% alcohol then at least 3 times with warm physiological saline (0.9% NaCl) containing 100 IU penicillin sodium salt and 100 µg/ml streptomycin. Cumulus-oocyte complexes (COCs) were aspirated from 3-8 mm follicles. COCs were collected under stereomicroscope at 20x then evaluated under stereomicroscope at 90x (Based on the number of cumulus-cell layers homogenous cytoplasm), COCs with more than 3 layers of cumulus cells and homogenous cytoplasm were used in the present experiment. COCs were washed 3 times in maturation medium which consisted of TCM-199 supplemented 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulation hormone (FSH) + 10 IU/ml human chorionic gonadotropin (HCG, El Nile Comp, Egypt) + 50 µg/ml gentamicin. In order to investigate the direct effect of glucocorticoid DEX on IVM of buffalo oocytes, buffalo cumulus-oocytes-complexes (COCs) were subjected to different concentrations of DEX (0, 1.0, 10, 50, 100 µg/ml) by adding DEX to maturation medium at the beginning IVM. COCs were cultured for IVM in 35 mm culture dish (Nunc, Denmark) at 38°C and under 5% CO₂ in humidified air for 24 h.

Assessment of Nuclear Maturation of *in vitro* Matured Buffalo Oocytes:

After 24 h of IVM, oocytes were checked for nuclear maturation. Cumulus cells were removed by gentle pipetting, oocytes were fixed in Acetic acid: ethanol (1:3 v/v) for 24 h then stained with 1% aceto-orceine stain for evaluation of nuclear configuration which was examined according to number of oocytes reaching the metaphase II (M II) stage as indicated by extrusion of 1st polar body, oocytes with polar body were considered as mature and that without polar body were considered as immature or degenerated according to their cytoplasm.

Experiment 2: Effect of Exposure to Dexamethasone During IVM on the Subsequent *In vitro* Fertilization Rate of Buffalo Oocytes

***In vitro* Maturation:** COCs were washed 3 times in maturation medium which consisted of TCM-199 supplemented with 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulation hormone (FSH) + 10 IU/ml human chorionic gonadotropin (hCG, El Nile Comp, Egypt) + 50 µg/ml gentamicin in the presence of 1.0, 10.0, 50.0 and 100.0 µg/ml DEX (experimental groups) or 0.0 control (control group). COCs were cultured in 35 mm culture dish (Nunc, Denmark) at 38°C and under 5% CO₂ in humidified atmosphere for 24 h.

***In vitro* Fertilization:** Buffalo frozen semen straws from the same batch of the same bull were used in the present work. Frozen semen straws were thawed in water bath at 37°C for 45 second and then layered on the top of 2 layers of Percoll density gradient (2 ml of 45% Percoll at the top and 2 ml of 90% Percoll at the bottom). After that the tube was centrifuged at 500 g for 30 minutes. The supernatant was removed and the sperm pellet was washed twice with 5 ml sperm Tyrod's Albumin Lactate Pyruvate medium (Sp-TALP) supplemented with 4 mg/ml BSA + 50 µg/ml gentamicin and centrifuged again for 5 minutes. The supernatant was removed and the pellet was re-suspended into 1 ml of Fertilization TALP (Fert-TALP) medium supplemented with 10 µg/ml heparin + 2.5 mM caffeine + 5 mg/ml BSA-FAF (Fatty acid free) plus 50 µg/ml gentamicin and the sperm concentration was adjusted to 1-2 x 10⁶ /ml. For fertilization 300 µl of sperm suspension was allocated into 4-wells culture dish

(Nunc, Denmark) and then covered with 200 µl of sterile paraffin oil. A total number of 15-20 matured buffalo oocytes were added to each well. Co-incubation of oocytes and spermatozoa was performed at 38.5°C under 5% CO₂ in humidified air for 18-20 h.

Assessment of Fertilization: After 18 h of oocyte-sperm co-incubation, the presence of 2 polar bodies was detected under stereomicroscope at 90x as evidence for fertilization.

Statistical Analysis: Data were statistically computed using SPSS20. Results were pooled and analyzed by *Chi square* test [12].

RESULTS

Experiment (1):- Effect of DEX on IVM Rate of Buffalo

Oocytes: The effect of addition of different concentrations of DEX to IVM culture medium on nuclear maturation of buffalo oocytes is illustrated in Figure 2. Data analysis revealed that the percentage of oocytes at GV stage (Immature oocytes) was not significantly different between control group and the treated one. However, the percentage of buffalo oocytes reaching metaphase II stage (M II, Fig. 1A) was significantly higher ($P<0.05$) in control than DEX treated groups and in a dose dependent manner. Our results revealed that DEX even at the lowest concentration (1.0 µg/ml) produced spontaneous oocyte activation (Presence of two polar bodies or spontaneously divided oocytes, Fig. 1B)

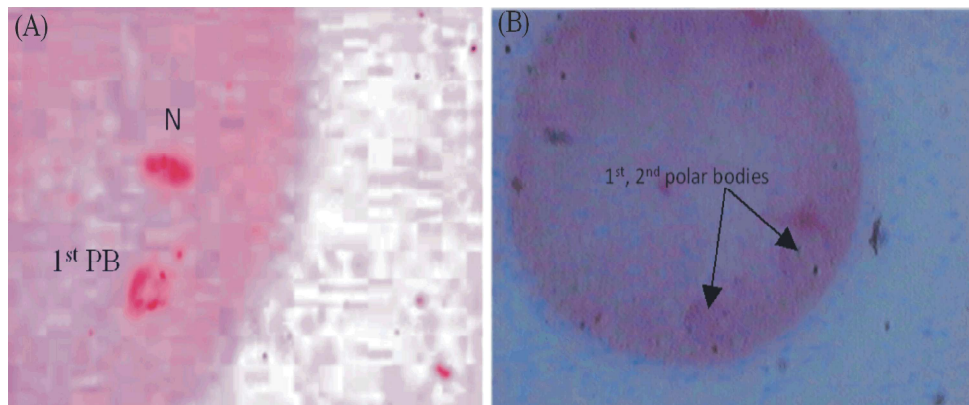


Fig. 1: Photograph showing mature M II buffalo oocytes (A) matured under control conditions and illustrating the nucleus (N) and first polar body (1st PB). Photograph showing activated buffalo oocytes (with 2 polar bodies) *in vitro* matured in the presence of DEX (B)

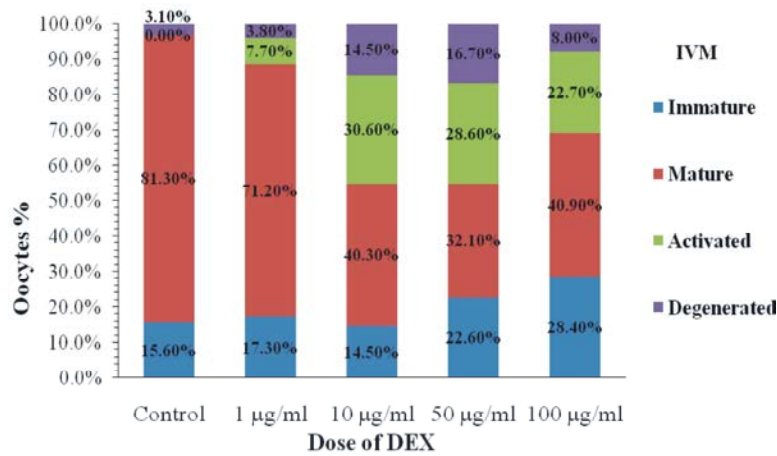


Fig. 2: Histogram representseffect of addition of different concentrations of DEX to TCM-199 on *in vitro* maturation rate of buffalo oocytes.

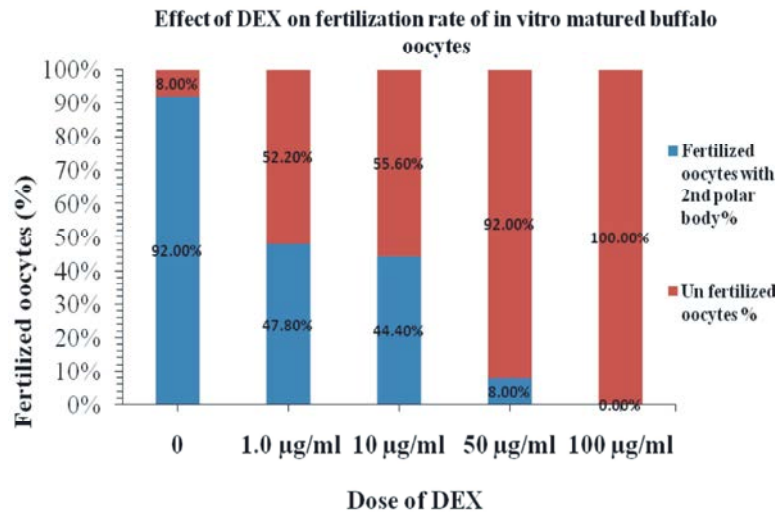


Fig. 3: Histogram represents effect of different concentrations of DEX on *in vitro* fertilization rate of buffalo oocytes.

Experiment (2):-Effect of dexamethasone on *In vitro* Fertilization (IVF) of Buffalo Oocytes: In the present study we investigated the effect of addition of different concentrations of DEX (0, 1.0, 10, 50, 100 µg/ml) on fertilization rate of *in vitro* mature buffalo oocytes. Cleavage was assessed at 48 h post fertilization. Results revealed that fertilization rate was significantly higher ($P<0.05$) in control compared with the DEX treated groups in a dose dependent manner.

DISCUSSION

In the present study we investigated the effect of the synthetic glucocorticoid DEX which is used largely in treatments of human being and animals, a long-acting synthetic glucocorticoid and the most potent synthetic

GCs (It has 25-50 times the glucocorticoids potency of cortisol), on bovine (Buffalo) female fertility using an *in vitro* model.

Our results demonstrated that DEX significantly decreased maturation rate of *in vitro* matured buffalo oocytes in a dose dependent manner. Similarly, Yang *et al.* [2] demonstrated that glucocorticoids directly inhibit the meiotic but not cytoplasmic maturation of pig oocytes *in vitro* in a concentration- and time-dependent manner, and they found that glucocorticoids inhibited nuclear membrane breakdown. In another study, Andersen [5] found that cortisol and dexamethasone did not affect meiosis resumption and maturation rate of mouse oocytes undergoing FSH-induced and spontaneous IVF. Since oocytes containing one polar body and a metaphase plate were regarded as matured,

the observation that glucocorticoids suppressed the rate of maturation of pig oocytes suggests that glucocorticoids may prevent chromatin condensation or reorganization of the cytoskeleton [2]. In contrast, Van *et al.* [4] showed that DEX has no influence on the regulation of oocyte nuclear maturation in mouse. Also, Gonzalez *et al.* [7] indicated that high glucocorticoid levels may have consequences for subsequent development, although a short exposure to physiologic or stress-related glucocorticoid levels may not represent a hazard to meiosis progression of mouse oocyte. This discrepancy could be due to species difference, dose of DEX used or the root of exposure.

Our study showed that buffalo oocytes which matured *in vitro* in presence of DEX showed spontaneous parthenogenetic activation as indicated by the presence of 2nd polar body. Moreover, some oocytes were spontaneously divided to four cells stage (parthenogenetically activated). Similarly, Gonzalez *et al.* [8] found that oocytes matured in presence of DEX were parthenogenetically activated at the end of incubation period and some oocytes displayed pronuclei but no parthenogenetic cleavage was observed at this time. Itagaki *et al.* [13] suggest that DEX increases calcium concentrations in *in vitro* cultured myocytes. In all mammalian species studied so far, oocyte activation is triggered by repetitive rises in the intracellular concentration of free Ca²⁺ ([Ca²⁺]_i) [14]. The [Ca²⁺]_i rises are generated by release of Ca²⁺ from the intracellular stores, which is mediated by production of inositol 1,4,5-triphosphate (IP₃) following activation of the phosphoinositide signaling pathway [15, 16]. So, as DEX increases intracellular Ca²⁺ which leading to cyclin degradation and so inactivation of MPF which means release of meiotic arrest and oocyte activation.

Our results revealed that DEX significantly affected the fertilization rate of *in vitro* matured buffalo oocytes and this may be attributed to its primary effect on maturation which affected subsequent fertilization. The maturation activating factor (MPF) complex together with the mitogen-activated protein kinases (MAPKs) play pivotal roles in regulating the maturation of the oocyte by an intricate and coordinated interplay including cross-talk with other components and cell signals [17, 18]. Gonzalez *et al.* [8] found a clear concentration-dependent inhibitory effect of cortisol and dexamethasone on both ERK-1 and ERK-2. MAPKs are activated by phosphorylation during oocyte maturation around the time of GVBD and their

activities remain at high levels until metaphase II, preventing parthenogenetic activation; after fertilization or parthenogenetic activation, MAPK becomes dephosphorylated resulting in loss of activity [19]. MAPK inactivation is required for normal spindle function and polar body emission [20] and it is associated with pronuclear formation [21]. They found decrease in phosphorylated active forms of ERK-1 and ERK-2 during oocyte incubation in the presence of glucocorticoids, which could explain the increase in parthenogenetic activation which was in agreement with our results.

In conclusion, our study shows that the synthetic glucocorticoid DEX affected buffalo female fertility by its direct effect on oocyte maturation and the subsequent fertilization.

REFERENCES

1. Harlow, C.R., J.M. Jenkins and R.M.L. Winston, 1997. Increased follicular fluid total and free cortisol levels during the luteinizing hormone surge. *Fertil Steril*, 68: 48-53.
2. Yang, J.G., W.Y. Chen and P.S. Li, 1999. Effects of glucocorticoids on maturation of pig oocytes and their subsequent fertilizing capacity *in vitro*. *Biol. Reprod*, 60: 929-936.
3. Chen, W.Y., J.G. Yang and P.S. Li, 2000. Effect of dexamethasone on the expression of p34^{cdc2} and cyclin B1 in pig oocytes *in vitro*. *Mol Reprod Dev.*, 56: 74-79.
4. Van, M.V., W.K. Van and R. Cortvrindt, 2007. *In vitro* effects of dexamethasone on mouse ovarian function and pre-implantation development. *Reprod. Toxicol.*, 23(1): 32-41.
5. Andersen, C.Y., 2003. Effect of glucocorticoids on spontaneous and follicle stimulating hormone induced oocyte maturation in mouse oocytes during culture. *The Journal of Steroid Biochemistry and Molecular Biology*, 85(2-5): 423-427.
6. Fateh, M., Z. Ben-Rafael, C.A. Benadiva, L. Mastroianni and G.L. Flickinger, 1989. Cortisol levels in human follicular fluid. *Fertil Steril*, 51: 538-541.
7. Gonzalez, R., Y.R. Leon, M. Gomendio and R.S. Roldan, 2010a. The effect of glucocorticoids on mouse oocyte *in vitro* maturation and subsequent fertilization and embryo development. *Toxicology in vitro.*, 24(1): 108-115.

8. Gonzalez, R., Y.R. Leon, M. Gomendio and R.S. Roldan, 2010b. The effect of glucocorticoids on ERK-1/2 phosphorylation during maturation of lamb oocytes and their subsequent fertilization and cleavage ability *in vitro*. *Reprod Toxicol.*, 29(2): 198-205.
9. Acosta, T.J., M. Tetsuka, M. Matsui, T. Shimizu, B. Berisha, D. Schams and A. Miyamoto, 2005. *In vivo* evidence that local cortisol production increases in the preovulatory follicle of the cow. *J. Reprod Dev.*, 51: 483-489.
10. Brien, T.G., 1988. Pathophysiology of free cortisol in plasma. *Ann NY Acad Sci.*, 538: 130-136.
11. Magiakou, M.A. and G.P. Chrousos, 1994. Corticosteroid therapy, non-endocrine disease and corticosteroid withdrawal. In: C.W. Bardwin, Editor, *Current therapy in endocrinology and metabolism* (5th ed.). Mosby Yearbook, Philadelphia, pp: 120-124.
12. Snedecor, G.E. and W.G. Gohran, 1982. *Statistical methods*. 6th Ed IOWA state university press, Ames IOWA USA, pp: 593.
13. Itagaki, K., M. Menconi, B. Antoniu, Q. Zhang, P. Gonnella, D. Soybel, C. Hauser and P.O. Hasselgren, 2010. Dexamethasone stimulates store-operated calcium entry and protein degradation in cultured L6 myotubes through a phospholipase A(2)-dependent mechanism. *Am J. Physiol Cell Physiol.*, 298(5): C1127-39.
14. Miyazaki, S.I., H. Shirakawa, K. Nakada and Y. Honda, 1993. Essential role of the inositol 1,4,5-triphosphate receptor/Ca²⁺ release channel in Ca²⁺ waves and Ca²⁺ oscillations at fertilization of mammalian eggs. *Dev. Biol.*, 158: 62-78.
15. Stith, B.J., M. Goalstone, S. Silva and C. Jaynes, 1993. Inositol 1,4,5-trisphosphate mass changes from fertilization through first cleavage in *Xenopus Laevis*. *Mol. Biol. Cell*, 4: 435-443.
16. Turner, P.R, M.P. Sheetz and L.A. Jaffe, 1984. Fertilization increases the polyphosphoinositide content of sea urchin eggs. *Nature.*, 310: 414-415.
17. Fan, H.Y. and Q.Y. Sun, 2004. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod.*, 70: 535-547.
18. Liang, C.G., Y.Q. Su, H.Y. Fan, H. Schatten and Q.Y. Sun, 2007. Mechanisms regulating oocytes meiotic resumption: roles of mitogen-activated protein kinase. *Mol. Endocrinol.*, 21: 2037-2055.
19. Liu, L. and X.Z. Yang, 1999. Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase-to-interphase transition of activated bovine oocytes. *Biol Reprod.*, 61: 1-7.
20. Phillips, K.P., M.A.F. Petrunewich, J.L. Collins, R.A. Booth, X.J. Liu and J.M. Baltz, 2002. Inhibition of MEK or cdc2 kinase parthenogenetically activates mouse eggs and yields the same phenotypes as Mos(-/-) parthenogenotes. *Dev Biol.*, 247: 210-23.
21. Moos, J., Z. Xu, R.M. Schultz and G.S. Kopf, 1996. Regulation of nuclear envelope assembly/disassembly by MAP Kinase. *Dev Biol.*, 175: 358-61.