# Teratogenic Effects of Metformin Hydrochloride on Developing Chick Embryo

**Kotchakorn Siripattanaphol[1*], Thanaporn Rungruang[1], Vasana Plakornkul[1], Yadaridee Viravud[1] and Jantima Roongruangchai[1]**

Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University (kchakorns@gmail.com)

---

## ABSTRACT

Metformin (MET), a natural product from *Galega officinalis linn*, has a biguanide component that can treat gestational diabetes mellitus (GDM), type 2 diabetes mellitus (T2DM) and polycystic ovary syndrome (PCOS). Due to some evidence suggested that MET could pass through the placenta causing embryo in risk. However, it was remained argument and had not adequate data for using control in the first trimester with PCOS pregnancy. The aim of this study was to evaluate the effects of MET in induced teratogenesis on the development of 72-hours chick embryos. The white leghorn hen eggs were divided into 4 groups; a control group injected with 0.9% normal saline and treated groups 2, 3 and 4 injected into the yolk sac with 0.1 ml of MET of 3, 4 and 5 mg/ml concentrations, respectively. The embryos were stained with Mayer's carmalum to determine of morphological observation. The results showed high mortality rate in all treated groups, especially in MET 4 mg/ml group (63.33% mortality) and 5 mg/ml group (58.62% mortality). The effects of MET displayed a delay in closure of anterior and posterior neuropores leading to form abnormalities brain and retard development of brain vesicle, eye, heart looping (U-shaped), branchial arches, somites and limb buds. In conclusion, the increasing MET concentration caused increasing the risk of mortality, retardation and malformations of early stage chick embryonic development. Hence, the importance of using MET to treat the first trimester pregnancy with diabetes should be considered as the standard of care.

**Keywords:** Chick Embryo, Gestational Diabetes Mellitus, Metformin, Pregnancy, Teratogenesis

## 1. Introduction

Gestational diabetes mellitus (GDM) is glucose intolerance (diabetogenic environment) with onset during pregnancy. GDM occurs when glucose metabolism cannot compensate and progressively develop insulin resistance, increase diabetogenic placental hormonal release (Singh & Singh, 2015). The prevalence of GDM worldwide has been reported between 9.2% and 45.3% of pregnancies (Agarwal, Dhatt & Othman, 2015). In Thailand, the Siriraj hospital and the Maharaj Nakorn Chiang Mai hospital were found the prevalence rates of GDM varies from 2.10% to 7.05% in pregnancy and trend to increase in obesity disease. Moreover, the women with GDM had a chance to develop which result in type 2 diabetes mellitus (T2DM), neonatal death, congenital defects,

and hypoglycaemia in neonates (Boriboonhirunsarn, Sunsaneevithayakul, & Nuchangrid, 2004; Chanprapaph & Sutjarit, 2004; Singh & Singh, 2015).

Metformin hydrochloride (MET) is a class of biguanide which commonly used to treat GDM, T2DM and polycystic ovary syndrome (PCOS) during pregnancy and no evidence for short-term fetal adverse outcomes. MET is a pregnancy category B drug - indicates to failed of prediction a risk to the fetus. However, the studies of its effect on pregnancy were limited and controversial. Lee and co-workers reported not sufficient for control using in the first trimester with PCOS pregnant women related to survival birth rate. Moreover, it may lose weight gain of women pregnancy, in contrast, the infants in gestational age are lower dangerous of neonatal hypoglycaemia. (Lee, Wei, & Loeken, 2014; Priya & Kalra, 2018; Vanky et al., 2005). Nevertheless, A study in patient with GDM showed that MET was transferred from pregnant women to embryo via the placenta (Nanovskaya et al., 2006). This information is indicated that using MET may cause harm to the embryo. Jacob (2018) demonstrated that the MET administration at a concentration of 1 μg/L caused an increase of the hepatic glycogen in brown trout (*Salmo trutta* f. *fario*) and a decrease in the bodyweight of fish. In 1979, Coetzee and Jackson (1979) suggested that pregnancy with GDM was revealed an incidence of 9% congenital abnormalities, 1 sacral agenesis, and 2 heart defects, on 3 infants. Hence, MET treatment may be a risk to perinatal mortality in GDM pregnant patients.

In our study, we evaluated the effect of MET on chick embryonic development to confirm the safety in therapeutic use of MET in pregnancy.

**2. Objectives of the study**

This study was estimated the effects of antidiabetic drug MET induced teratogenesis on the development of 72-hour chick embryos by observation of the morphology using total mount preparations.

**3. Materials and methods**

Material preparations

Metformin hydrochloride (MET) 500 mg was purchased from a government pharmaceutical organization (GPO, Thailand). The substance was soluble in 50 ml sodium chloride 0.9% (normal saline; NSS), purchased from the drugstore (Pharma Innova, Thailand). In this study, MET was diluted to be as 3, 4 and 5 mg/ml concentrations, respectively.

*In ovo* protocol

For this *ovo* study used the fertilized chicken eggs (*Gallus gallus domesticus*) were purchased from Suwanvajokkasikij Animal Research and Development Institute, Kasetsart University. Before incubating at 37-39°C, 50-56% humidity, eggshells were cleaned with 70% ethanol (EtOH). These fertilized chicken eggs were randomly and blindly divided into 4 groups including control and 3 MET treated groups. A control group (N = 9)

received 0.9% NSS and groups 2 (N = 9), 3 (N = 6) and 4 (N = 6) were injected into the yolk sac with MET of 3, 4 and 5 mg/ml concentrations, respectively (Figure 1).

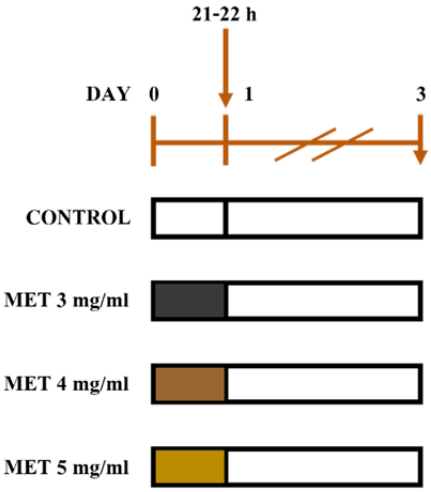


**Figure 1.** Experimental design, the control group was received 0.9% NSS injection. The embryonic egg group 2,3 and 4 were administered with 3, 4 and 5 mg/ml concentrations, respectively. There were injected during 21-22 h after incubation.

After incubation of the eggs on day 3 (72 h), they were evaluated for survival and mortality rate. All survival embryos were washed with 0.9% NSS and fixed in formaldehyde acetic acid (FAA), then processed for the total mount method. The total mounts were stained with Mayer's carmalum to evaluated morphological observation. The malformations and survival and mortality rate were recorded and compared to the control group.

Light Microscope

The stained 72 h chick embryos were examined under an Olympus BX41 light microscope and recorded with digital camera software for DP70 with U-TV0.5XC-3 adaptor.

**4. Results**

The survival and Mortality rates of 72-hours chick embryos

The survival and mortality rates were observed of the heart palpitation and blood circulation. The control group has presented of 95% survival. On the contrary, all experimental groups were shown higher mortality rates than control group (Table 1).

**Table 1** The percentages of survival and mortality rates of 72-hours chick embryos of 4 groups, including control, and treated with 3, 4 and 5 mg/ml MET.

| Group | N | % survival | % Mortality |
|---|---|---|---|
| Control | 20 | 95% (19) | 5% (1) |
| MET 3 mg/ml | 30 | 53.33% (16) | 46.67% (14) |
| MET 4 mg/ml | 30 | 36.67% (11) | 63.33% (19) |
| MET 5 mg/ml | 29 | 41.38% (12) | 58.62% (17) |

Total mount

The control group (N = 9) (Figure 2A) showed normal developmental character as stage 19 (during 68-72 h) follow described by Hamburger and Hamilton. Chick embryos have appeared 36 somites extended from the cervical flexure to the caudal end, the embryo turned to the left side and the caudal end of embryo twisted 90 degrees. The cephalic and cervical flexures were almost completed. There was the presence of 5 brain vesicles, including telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. The horse-shoe shaped optic cup was located at the lateral wall of the diencephalon and the lens vesicle was in the middle part of the optic cup. The large sac of otocyst which developed at the myelencephalon level was completely closed. There was the presence of 4 brachial arches. The S-shaped heart loop was below the branchial arches whereas the ventricle protruded from the trunk. Anterior and posterior limb buds were presented at the correct level.

The MET 3 mg/ml group (N = 9) (Figure 2B) showed retardation of embryonic development with the morphological similarly to stage 14. There was the disappearance of 5 brain vesicles with only cephalic flexure despite closed anterior neuropore. The optic vesicle invaginated to form the optic cup. Additionally, there was the absence of lens, branchial arches, and limb buds. Incomplete formation of otocyst and heart loop. Furthermore, the appearance of a parallel dense line on the somites but did not extend to the caudal end. Posterior neuropore was still opened.

The MET 4 mg/ml group (N = 6) (Figure 2C) showed retardation development which was similar to between embryonic stages 14 and 15 with some abnormalities, including the opening of anterior neuropore caused unidentified brain structure and ambiguous of cephalic flexure. Primordial eye and limb buds were absent. Additionally, incomplete formations of otocyst and branchial arches, and abnormally dilated heart loop were observed. The opening of the posterior neuropore was opened.

A final group of embryos exposed to MET at a concentration of 5 mg/ml (N = 6) (Figure 2D) showed retardation of brain vesicle with only cephalic flexure which was similar to stage 14. The optic vesicle was on the cephalic part and small otocyst was formed. There was the absence of branchial arches and limb buds. Abnormally

dilated heart loop and non-extended somites to caudal end were observed. Anterior and posterior limb buds were disappeared.

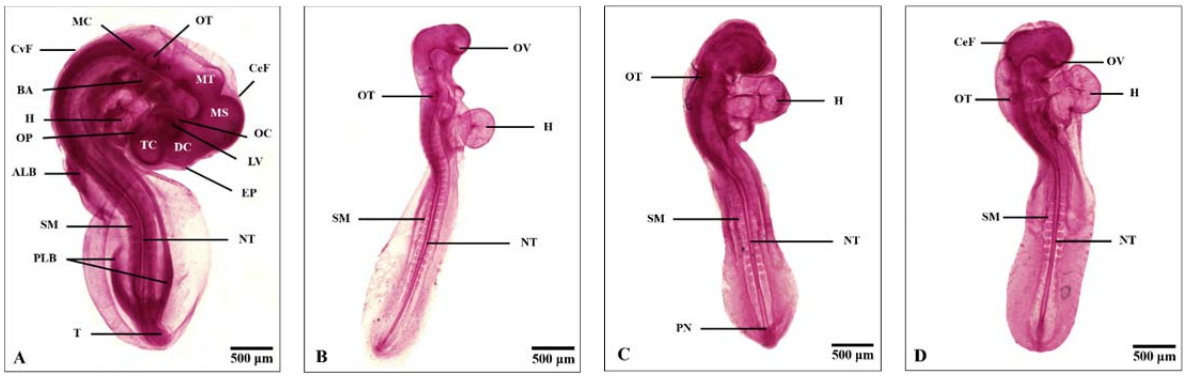


**Figure 2.** Micrographs showed total mount of 72-hours chick embryo of the control group (A), MET 3 mg/ml (B), MET 4 mg/ml (C) and MET 5 mg/ml (D). TC: Telencephalon, DC: Diencephalon, MS: Mesencephalon, MT: Metencephalon, MC: Myelencephalon, LV: Lens vesicle, OC: Optic cup, OV: Optic vesicle, OT: Otocyst, CeF: Cephalic flexure, CvF: Cervical flexure, BA: Branchial arch, H: Heart, ALB: Anterior limb bud, PLB: Posterior limb bud, SM: Somite, NT: Neural tube, T: Tail

**5. Discussion**

In the first trimester of pregnancy, mothers can have a situation of gestational diabetes mellitus (GDM) and develop to type 2 diabetes mellitus (T2DM) (Singh & Singh, 2015). In this study, retardation in the nervous system were observed in the experimental groups which is comprehended to the study of Balsells and collegues in 2012. They described that the women with GDM are implicated with an increased risk of congenital malformations especially, the neural tube defects (Balsells et al., 2012).

About the mortality rate in this study showed that the mortality rates in all experimental groups were higher than those of control. The survivors have shown retardation and malformations.

The morphology of developing embryo observed in the control group appeared normal development with the characteristic similarly with stage 18 as described by Hamburger and Hamilton (1992). MET treated groups revealed abnormalities of brain and retardation of eye, otocyst, heart, branchial arches, somites, absence of limb buds, and opening of neuropores. Similar to the study by Denno and Sadler (1994) showing the administration of MET at doses from 0.15 to 1.8 mg/ml after 24 h culture in mice caused 9-16% failure of neural tube closure. Conversely, Shepard and Schardein suggested that MET did not show teratogen in rat fetuses, nonetheless, the administration of MET in high dose was shown embryotoxicity incidence (Briggs, Freeman & Yaffe, 2008). However, this study can be improved by further study with more sample to obtain an accurate results and statistical analysis is needed to evaluate and conclude the key finding in this study.

**6. Conclusion**

In conclusion, our study revealed using high dose of MET concentrations (MET 4 and 5 mg/ml) may trend to the risk of lethal or embryotoxic effects in chick embryos. Furthermore, the results showed retardation and malformations in early-stage chick embryos development. Therefore, using MET in pregnancy with GDM or PCOS in the first-trimester should appropriately control of dose before treatment.

**Acknowledgements**

This work is a part of the project in graduate study (master's degree). The author sincerely thank you for support from the research advisor, co-advisors, and staff at the Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University.

**References**

Agarwal, M. M., Dhatt, G. S., & Othman, Y. (2015). Gestational diabetes: Differences between the current international diagnostic criteria and implications of switching to IADPSG. *Journal of Diabetes and Its Complications*, *29*(4), 544–549. https://doi.org/10.1016/j.jdiacomp.2015.03.006

Balsells, M., García-Patterson, A., Gich, I., & Corcoy, R. (2012). Major congenital malformations in women with gestational diabetes mellitus: a systematic review and meta-analysis. *Diabetes/Metabolism Research and Reviews*, *28*(3), 252–257. https://doi.org/10.1002/dmrr.1304

Boriboonhirunsarn, D., Sunsaneevithayakul, P., & Nuchangrid, M. (2004). Incidence of Gestational Diabetes Mellitus Diagnosed Before 20 Weeks of Gestation - PubMed. *The Medical Association of Thailand*, *87*(9), 1017–1021. Retrieved from https://pubmed.ncbi.nlm.nih.gov/15516000/

Briggs, G. G., Freeman, R. K., & Yaffe, S. J. (2008). *Drugs in Pregnancy and Lactation: A Reference Guide to Fetal and Neonatal Risk* (8th ed.). Philadelphia: Lippincott Williams & Wilkins. Retrieved from https://books.google.co.th/books?id=YOEV2w3XTxsC&pg=PA1156&lpg=PA1156&dq=Shepard+and+Schardein+metformin+teratogen&source=bl&ots=Ztuf21t7qk&sig=ACfU3U37tuSOx6kaRmnpjX4e0E9wqgnvCw&hl=en&sa=X&ved=2ahUKEwiG7Pad2-_pAhVaT30KHf_-A_8Q6AEwAHoECAsQAQ#v=onepage&q=S

Chanprapaph, P., & Sutjarit, C. (2004). Prevalence of Gestational Diabetes Mellitus (GDM) in Women Screened by Glucose Challenge Test (GCT) at Maharaj Nakorn Chiang Mai Hospital - PubMed. *The Medical Association of Thailand*, *87*(10), 1141–1146. Retrieved from https://pubmed.ncbi.nlm.nih.gov/15560687/

Coetzee, E. J., & Jackson, W. P. U. (1979). Metformin in management of pregnant insulin-independent diabetics. *Diabetologia*, *16*(4), 241–245. https://doi.org/10.1007/BF01221950

Denno, K. M., & Sadler, T. W. (1994). Effects of the biguanide class of oral hypoglycemic agents on mouse embryogenesis. *Teratology*, *49*(4), 260–266. https://doi.org/10.1002/tera.1420490405

Hamburger, V., & Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo.

*Developmental Dynamics*, *195*(4), 231–272. https://doi.org/10.1002/aja.1001950404

Jacob, S., Dötsch, A., Knoll, S., Köhler, H.-R., Rogall, E., Stoll, D., … Triebskorn, R. (2018). Does the antidiabetic drug metformin affect embryo development and the health of brown trout ( Salmo trutta f. fario )? *Environmental Sciences Europe 2018 30:1*, *30*(1), 1–16. https://doi.org/10.1186/S12302-018-0179-4

Lee, H. Y., Wei, D., & Loeken, M. R. (2014). Lack of metformin effect on mouse embryo AMPK activity: implications for metformin treatment during pregnancy. *Diabetes/Metabolism Research and Reviews*, *30*(1), 23–30. https://doi.org/10.1002/dmrr.2451

Nanovskaya, T. N., Nekhayeva, I. A., Patrikeeva, S. L., Hankins, G. D. V., & Ahmed, M. S. (2006). Transfer of metformin across the dually perfused human placental lobule. *American Journal of Obstetrics and Gynecology*, *195*(4), 1081–1085. https://doi.org/10.1016/j.ajog.2006.05.047

Priya, G., & Kalra, S. (2018). Metformin in the management of diabetes during pregnancy and lactation. *Drugs in Context*. Bioexcel Publishing LTD. https://doi.org/10.7573/dic.212523

Singh, A., & Singh, R. (2015, March 1). Metformin in gestational diabetes: An emerging contender. *Indian Journal of Endocrinology and Metabolism*. Medknow Publications. https://doi.org/10.4103/2230-8210.149317

Vanky, E., Zahlsen, K., Spigset, O., & Carlsen, S. M. (2005). Placental passage of metformin in women with polycystic ovary syndrome. *Fertility and Sterility*, *83*(5), 1575–1578. https://doi.org/10.1016/j.fertnstert.2004.11.051