**STUDI MOLEKULAR TERJADINYA CELAH PALATUM (*Cleft Palate*) AKIBAT PEMBERIAN DIAZEPAM PADA MENCIT PRENATAL MELALUI EKSPRESI GABA, CASPASE-9, BAX DAN APOPTOSIS**

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**DIAZEPAM ; APOPTOSIS**
**KKA KK Dis K 25 / 12 Yul s**


## ABSTRACT

**MOLECULAR STUDY OF OCCURRENCE CLEFT PALATE DUE TO DIAZEPAM INTAKE IN MICE PRENATAL THROUGH EXPRESSION OF GABA, CASPASE-9, BAX AND APOPTOSIS**

This study aims to explain the occurrence of cleft palate in the embryos of mice due to intake of diazepam in the period of organogenesis by analyzing changes in GABA levels and expression, protein expression of caspase-9, Bax and Apoptosis.
About 27 mice of known age and weight were used. After pregnancy, they were divided in three groups: the first groups were control group which were injected daily with distilled water. The second groups that were injected daily Diazepam 8 mg/kg/day and the third injected daily with Diazepam 16 mg/kg/day. All the above groups were administered intraperitoneally injection during the organogenesis phase ($9^{th}$ -$15^{th}$ days) On the $15^{th}$ of gestation period all the pregnant mice were sacrificed and the embryos were studied macroscopically for anomalies and then the tissue were fixed and processed, stained and examined microscopically. Anomalies of cleft palate were evaluated. Apoptotic cells in tissue section were identified by TUNEL Assay and for expression of GABA, caspase-9 and Bax were identified by immunohistochemistry. Examination GABA levels by ELISA and protein expression of caspase-9 and Bax were observed using SDS-PAGE and Western Blotting.
The results of the detailed microscopic analyses on the embryos of the first and second test groups show that the cleft palate appeared to be a complete failure of the second palate formation. There are significant differences ($p < 0.05$) on all variables observed between control and treatment groups except the expression of GABA test group I and controls (0.084). Increased GABA levels significantly in both test groups. Protein caspase-9 and Bax was detected specifically in the range of molecular weight of 49 kDa and 20 kDa. Conclusion: The exposure to diazepam during the period of organogenesis causes *cleft palate*, with inhibition of shelves elevation caused an increase GABA and apoptosis activity through the intrinsic apoptosis path.

**Keywords**: diazepam, cleft palate, apoptosis, GABA, caspase-9, Bax, malformation

# RINGKASAN

## STUDI MOLEKULAR TERJADINYA CELAH PALATUM (C*left Palate*) AKIBAT PEMBERIAN DIAZEPAM PADA MENCIT PRENATAL MELALUI EKSPRESI GABA, CASPASE-9, BAX DAN APOPTOSIS

Diazepam merupakan senyawa psikoaktif, prototip dari obat sedatif golongan benzodiazepin, yang digunakan secara luas, namun penggunaannya selama kehamilan menjadi kontroversi karena diduga menyebabkan malformasi congenital yaitu celah palatum (*cleft palate*) (Marinucci, 2008 ; Igbal *et al*, 2002). Efek diazepam dan sedatif-hipnotik lainnya, seperti mengatasi ansietas, *euphoria* dan mempercepat tidur, menyebabkan kemungkinan terjadinya penyalahgunaan obat (*drug abuse*) sehingga perlu menjadi perhatian khusus. Celah palatum (*Cleft Palate*) merupakan salah satu malformasi kongenital yang paling sering terjadi di dunia dengan karakteristik cacat atau kelainan pada dinding atas rongga mulut, dengan dampak sosial, psikologis dan medis (Iseki *et al*, 2007; Meng *et al*., 2009). Cacat jenis ini merupakan urutan tertinggi dari seluruh jenis cacat bawaan lahir yang dikenal pada penduduk dunia dengan angka rata-rata 1 kejadian per 700 kelahiran (Kerrigan *et al*, 2000). Etiologinya sangat kompleks dan multifaktorial, baik faktor genetik maupun faktor lingkungan terlibat dan diregulasi melalui mekanisme molekular yang sangat rumit (Shengjun *et al*, 2008).. Kegagalan pembentukan palatum sekunder, lebih sering menimbulkan kejadian celah palatum. Pembentukan palatum sekunder merupakan suatu rangkaian kejadian dalam perkembangan embrio yang terjadi pada seluruh mamalia, terjadi melalui serangkaian proses yang rumit dan bervariasi antar kelas. *Shelf elevation* dan *palatal fusion* adalah dua tahap paling kritis dari proses palatogenesis, (Iseki *et al*, 2007; Sadler, 2000; Gritli-Linde, 2007, Hutahean, 2004) (Sheng-jun *et al*, 2008). Pada proses penyatuan lempeng pembentukan palatum sekunder (*secondary palate shelt fusion*) dibutuhkan program kematian sel (apoptosis) dan proses ini dapat dipengaruhi oleh zat teratogen (Cuervo *et al*, 2002; Gritli-Linde, 2007). Efek teratogenik diazepam mengakibatkan terjadinya celah palatum (*cleft palate*), diduga akibat aktivitas farmakologi diazepam pada reseptor *translocator protein* (TSPO), yang sebelumnya disebut reseptor benzodiazepin perifer (*The peripheral benzodiazepine receptor*) atau reseptor benzodiazepin mitokondria (*The mitochondrial benzodiazepine receptor*), yang berperan dalam inisiasi dan regulasi apoptosis. Aktivitas tersebut dapat memicu aktivasi apoptosis, khususnya apoptosis jalur intrinsik. Jalur ini dipengaruhi keseimbangan factor pro dan antiapoptotik, dimana protein Bax merupakan salah satu factor pro-apoptotik yang akan menyebabkan pelepasan sitokrom c yang dapat menginduksi aktivasi caspase inisiator dan caspase efektor, sehingga menyebabkan terjadinya apoptosis. Caspase-9 merupakan caspase inisiator utama yang menjadi salah satu parameter uji dalam penelitian ini. Selain itu efek teratogenik diazepam juga berkaitan dengan kerja diazepam pada *gamma-aminobutyric acid receptor* (GABRB3) yang mempunyai implikasi dalam regulasi pembentukan palatum. GABRB3 merupakan reseptor dari neurotransmitter *Gamma-aminobutyric acid* (GABA), suatu neurotransmitter inhibitori utama pada sistem saraf pusat.

Tujuan penelitian ini adalah menjelaskan mekanisme molekular terjadinya celah palatum pada embrio mencit akibat pemberian diazepam di periode dari dan jumlah sel yang mengekspresikan GABA, jumlah sel yang mengekspresikan protein Caspase-9 dan Bax, dan jumlah sel yang mengalami apoptosis. Rancangan penelitian ini adalah penelitian eksperimental murni, dengan sampel mencit dewasa, bunting, berat badan 20-40 gram dan embrio mencit yang diperoleh pada gestasi hari ke 15. Sampel dibagi menjadi tiga kelompok yaitu kelompok kontrol, Uji I dan Uji II. Masing-masing kelompok 9 ekor. Kelompok kontrol diberi injeksi larutan fisiologis, kelompok uji I, diberi injeksi diazepam 8 mg/kgBB dan kelompok uji II diberi injeksi diazepam 16 mg/kgBB. Semua perlakuan diberikan secara intraperitonial dimulai pada gestasi hari ke 9 sampai hari ke 15. Setiap sampel jaringan palatum dibuat preparat histologis, diwarnai *haematoxylin-eosin* (HE). Dilakukan pemeriksaan imunohistokimia, menggunakan antibodi monoklonal terhadap GABA, Caspase-9 dan Bax. Pemeriksaan sel-sel yang mengalami apoptosis menggunakan *Tunel Assay*. Penilaian jumlah sel yang mengekspresikan GABA, protein Caspase-9 dan Bax dengan cara mengamati warna coklat pada sitoplasma dan apoptosis tampak warna coklat pada intisel, terhadap masing-masing *slide* pada bidang pandang dengan perbesaran 1000x dan sebanyak 20 lapang pandang. Pemeriksaan kadar GABA dilakukan menggunakan metode ELISA dan identifikasi protein spesifik dilakukan menggunakan metode SDS-PAGE dan Western Blotting. Berdasarkan uji Anova, terjadi peningkatan bermakna jumlah sel yang mengekspresikan GABA pada kelompok uji II, jumlah sel yang mengekpresikan protein Bax pada kedua kelompok uji, dan terdapat perbedaan bermakna antara kedua kelompok uji. Jumlah se yang mengekspresikan protein caspase-9 kedua kelompok uji juga menunjukkan peningkatan signifikan tapi tidak ada perbedaan bermakna antara kedua kelompok. Rerata jumlah sel yang mengalami apoptosis pada kedua kelompok uji juga menunjukkan perbedaan signifikan dan terdapat perbedaan bermakna antara kedua kelompok uji. Terdapat perbedaan bermakna pada pengukuran kadar GABA pada kedua kelompok perlakuan, serta teridentifikasi protein spesifik caspase-9 pada 49 kDa dan Bax pada 20 kDa. Dari hasil penelitian ini dapat ditarik kesimpulan bahwa diazepam dapat menyebabkan peningkatan jumlah GABA, jumlah sel yang mengekspresikan GABA, protein Bax, protein Caspase-9 dan jumlah sel yang mengalami apoptosis, sehingga menginduksi terjadinya celah palatum (*cleft palate*). Dosis diazepam memiliki hubungan dengan terjadinya celah palatum. Manfaat penelitian ini secara teoritik adalah memberi informasi tambahan dalam pengembangan ilmu pengetahuan khususnya mekanisme teratogenik dan tingkat keamanan penggunaan diazepam. Secara praktis hasil temuan ini dapat digunakan oleh pihak terkait untuk meningkatkan kehati-hatian dalam penggunaan obat terutama pada obat-obatan yang memiliki kecendrungan untuk digunakan dengan salah (*drug misuse*) dan penyalahgunaan obat (*drug abuse*).

# SUMMARY

## MOLECULAR STUDY OF OCCURRENCE CLEFT PALATE DUE TO DIAZEPAM INTAKE IN MICE PRENATAL THROUGH EXPRESSION OF GABA, CASPASE-9, BAX AND APOPTOSIS

Cleft palate is one of the most frequent congenital malformations occur in the world with characteristic defects or abnormalities in the wall of the oral cavity, with the social, psychological and medical (Iseki et al, 2007; Meng et al., 2009). Defects of this type is the highest order of all types of birth defects known to the world population with an average rate of incidence per 700 births (Kerrigan et al, 2000). Very complex and multifactorial etiology, both genetic factors and environmental factors are involved and regulated through a very complicated molecular mechanism (Sheng-jun et al, 2008). Failure of secondary palate formation, more often cause cleft palate incidence. Palatal shelf elevation and fusion are the two most critical stage of the process palatogenesis, (Iseki et al, 2007; Sadler, 2000; Gritli-Linde, 2007, Hutahean, 2004), and occurs at the age of embryos varies among different species (Sheng-jun et al, 2008). In the process of unification of the plate forming a secondary palate (secondary palate fusion shelt) required programmed cell death (apoptosis) and this process can be affected by substances teratogens (Cuervo et al, 2002; Gritli-Linde, 2007). Teratogenic effect of diazepam resulted in cleft palate, allegedly due to pharmacological activity of diazepam in the receptor protein translocator (TSPO), previously called the peripheral benzodiazepine receptor (The peripheral benzodiazepine receptor) or the mitochondrial benzodiazepine receptor (the mitochondrial benzodiazepine receptor), which acts in the initiation and regulation of apoptosis. These activities can trigger the activation of apoptosis, particularly apoptosis intrinsic path. The line was influenced by the balance of pro and antifactor apoptotik, where the protein Bax is one factor for pro-apoptotik which will cause the release of cytochrome c can induce the activation of initiator caspase and effector caspase, thus causing the occurrence of apoptosis. Caspase-9 is the main initiator caspase which became one of the parameters tested in this study. In addition, teratogenic effects of diazepam also related to work on gammaaminobutyric acid receptor (GABRB3), which has implications in the regulation of palate formation. GABRB3 is the receptor of the neurotransmitter gammaaminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system. The purpose of this study is to explain the molecular mechanism of cleft palate in mice embryonic caused by diazepam induction during period of organogenesis by differences in expression of GABA, Bax, caspase-9 and cells undergoing apoptosis. The study is designed purely experimental, with samples of adult mice, pregnant, weight 20-40 grams and embryos of mice obtained on gestation day 15. Samples were divided into three groups: control group, Test I and Test II. Each group consists of nine mice. The control group was given injections of physiological solution, the test group I, given a diazepam injection 8 mg / kg and xii the test group II was given injection diazepam 16 mg / kg. All treatments administered intraperitoneally beginning on gestation day 9 until day 15. Embryos were treated with fixation solution for 24 hours. Samples were

put in paraffin after fixation and undergone tissue passage with H & E. They were then studied under light microscopy for anomalies. Immunohistochemistry examined using monoclonal antibodies against GABA, caspase-9, Bax. TUNEL staining is used to detect undergone apoptosis. GABA expression, caspase-9 and Bax assessed by observing the brown color in the cytoplasm and apoptosis appear on nucleus brown color, on each slide in the field of view with a magnification of 1000x on 20 fields of views. GABA levels examined using ELISA method and identification of specific proteins performed using SDS-PAGE and Western Blotting. Based on ANOVA test, there was a significant increase in expression of GABA in the test group II and significant in expression of Bax in both test groups, but there were no significant differences between the two test groups. Expression of caspase-9 in both test groups, showing a significant improvement but no significant difference between the two test groups. The mean expression of apoptosis in both test group also showing significant differences and there are significant differences between the two test groups. There are significant differences in the measurement of GABA levels in both treatment groups, and identified specific protein caspase-9 at 49 kDa, and Bax at 20 kDa. The results of this study can be concluded that diazepam can cause an increase in the number of GABA and the number of cells undergoing apoptosis, thus inducing the occurrence of cleft palate (cleft palate), the greater the dose, the more number of cells undergoing apoptosis. The benefit of this research is to theoretically provide additional information in the development of knowledge, especially the mechanism of teratogenic and security levels of diazepam usage. The practical result of these findings can be used by all relevant parties to increase the prudence in the use of drugs, especially in drugs that have a tendency to be drug misuse and drug abuse.