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Özgün Araştırma / *Research Article*

The Effects of Thyrotropin-Releasing Hormone, Glucocorticoids and Insulin on Experimentally Induced Diaphragmatic Hernia in Newborn Rats

Yenidoğan Ratlarda Deneysel Olarak Oluşturulan Diyafram Hernisi Üzerine Tirotropin Salgılatıcı Hormon, Glikokortikoid ve İnsülinin Etkileri

Ayşe Bahar Edirne[1], Berat Dilek Demirel[1], Ünal Bıçakcı[1], Yurdanur Süllü[2], Dilek Güvenç[3] Murat Fındık[3], Burak Tander[4]

[1]Ondokuz Mayıs Üniversitesi Tıp Fakültesi, Çocuk Cerrahisi Anabilim Dalı, Samsun, Türkiye
[2]Ondokuz Mayıs Üniversitesi Tıp Fakültesi, Tıbbi Patoloji Anabilim Dalı, Samsun, Türkiye
[3]Ondokuz Mayıs Üniversitesi Veterinerlik Fakültesi, Samsun, Türkiye
[4]Acıbadem Üniversitesi Tıp Fakültesi, Çocuk Cerrahisi Anabilim Dalı, İstanbul, Türkiye

ABSTRACT

Objective: *To evaluate the effects of thyrotropin-releasing hormone (TRH), glucocorticoids and insulin on experimentally induced diaphragmatic hernia.*
Method: *Congenital diaphragmatic hernia was induced by antenatal nitrofen administration. The offsprings of five pregnant rats were assigned into 5 groups; the control group (CG), the sham group (SG), the TRH group (nitrofen and TRH; TRHG), the glucocorticoid group (nitrofen and dexamethasone; GG) and the insulin group (nitrofen and insulin; IG). On gestational day 21, each mother rats gave birth to 7, 7, 8, 10 and 9 infants in the respective groups. After recording the Apgar scores, diaphragmatic defects were evaluated, lung tissues were weighed and histologically evaluated.*
Results: *The median Apgar scores in the groups were 5, 3, 4, 4 and 5, respectively. The mean Apgar score in the Group TRHG or IG was not statistically significantly different from that of the Group CG . In all groups, the number of defects in the diaphragm was less than those of the Group SG. The lowest defect rate (12.5%) was observed in the Group TRHG. The closest lung weight to CG was observed in IG. The mean numbers of alveoli and bronchi were highest in the Groups CG and IG.*
Conclusion: *Antenatal administration of insulin demonstrated significant improvements on Apgar scores, lung weights and the number of alveoli and bronchi of rats with diaphragmatic hernia. This study may lead to the new studies on antenatal insulin administration in diaphragmatic hernia.*

Keywords: *Congenital diaphragmatic hernia, nitrofen, insulin, dexamethasone, thyrotropin-releasing hormone*

ÖZ

Amaç: *Tirotropin salgılatıcı hormon (TRH), glukokortikoid ve insülinin deneysel olarak oluşturulan diyafram hernisi üzerine etkilerinin değerlendirilmesi.*
Yöntem: *Konjenital diyafram hernisi antenatal nitrofen uygulamasıyla oluşturuldu. Beş gebe rattan nitrofen verilmeyen ratın yavruları Kontrol Grubunu (KG), nitrofen verilen ratın yavruları Sham Grubunu (SG), nitrofen ve TRH verilen ratın yavruları TRH Grubunu (TRHG), nitrofen ve deksametazon verilen ratın yavruları Glukokortikoid Grubunu (GG) ve nitrofen ve insülin verilen ratın yavruları İnsülin Grubunu (IG) oluşturdu. Gestasyonun 21. gününde gruplarda sırasıyla 7, 7, 8, 10 ve 9 yavru doğdu. Yavru ratların Apgar skorları kaydedildikten sonra sakrifiye edilerek, diyafram defektleri değerlendirildi, akciğer dokuları tartıldı ve akciğer dokusu histolopatolojik olarak değerlendirildi.*
Bulgular: *Apgar skoru ortancası gruplarda sırasıyla 5, 3, 4, 4 ve 5'ti. Apgar skorlarında TRHG ve IG grupları ile CG arasında istatistiksel anlamlı fark yoktu. Diğer gruplarda diyafram defekt oranı SG Grubu'ndan daha azdı. En az diyafram defekti oranı (% 12.5) TRHG'de gözlendi. IG'deki ortalama akciğer ağırlığı CG'na en yakındı. Ortalama alveol ve bronş sayıları CG ve IG'de en yüksekti.*
Sonuç: *Antenatal insülin uygulaması, diyafragma hernisi olan sıçanların Apgar skorları, akciğer ağırlıkları, alveol ve bronş sayıları üzerinde anlamlı pozitif etkiler göstermiştir. Bu çalışma, diyafragma hernisinde antenatal insülin uygulamasıyla ilgili yeni çalışmalara öncül olabilir.*

Anahtar kelimeler: *Konjenital diyafragma hernisi, nitrofen, insulin, deksametazon, tirotropin salgılatıcı hormon*

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Berat Dilek Demirel
Ondokuz Mayıs Üniversitesi
Tıp Fakültesi Çocuk Cerrahisi
Anabilim Dalı
Samsun, Türkiye
bdayhan@hotmail.com
ORCID: 0000-0002-9358-0045

A.B. Edirne 0000-0001-9294-8146
Ü. Bıçakcı 0000-0002-9604-4184
Y. Süllü 0000-0002-8029-2490
D. Güvenç 0000-0003-0036-0914
M. Fındık 0000-0003-1408-2548
B. Tander 0000-0003-2013-0745

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Introduction

Congenital diaphragmatic hernia (CDH) is a congenital anomaly which is associated with a significant rate of mortality and characterised by translocation of the abdominal organs into the thoracic cavity due to a defect in the diaphragm. The resulting pulmonary hypoplasia (PH) and persistent pulmonary hypertension are the main causes of mortality [1]. Despite the increased availability of antenatal diagnostic tests, newborn intensive care units, high-frequency oscillatory mechanic ventilation and widespread use of nitric oxide have decreased mortality rates, CDH still remains as a significant source of mortality amd morbidity.

CDH has been investigated in several clinical and experimental studies [2-4]. Similar to observations in humans, CDH has been experimentally induced in newborn rats of female rats that were exposed to nitrofen [3]. A number of pharmacologic agents have been evaluated to prevent the development of diaphragmatic defect and PH in newborn rats with CDH induced by maternal exposure to nitrofen [4-6]. A study that evaluated the mechanism of the action of nitrofen in the development of CDH found lower levels of thyroid hormones in rats exposed to nitrofen [7]. The low levels of free triiodothyronine in the cord blood of newborns with transient tachypnea, suggested that thyroid hormones might be involved in the maturation of lung during antenatal period [8]. Another study suggested that antenatal administration of dexamethasone might prevent respiratory distress syndrome [9]. The increased incidence of respiratory distress syndrome in newborns of diabetic mothers, as well as the lower rates of respiratory problems in well-controlled diabetes suggest that insulin might be involved in the maturation of lung tissue during the antenatal period [10].

Antenatal treatment with thyroid hormones, glucocorticoids and insulin may be effective to prevent or alleviate PH, which is one of the major causes of mortality and morbidity in CDH. Therefore, the aim of the present study is to evaluate the effects of thyrotropin-releasing hormone (TRH), insulin and glucocorticoids on experimentally induced diaphragmatic hernia.

Materials and Methods

The current study was approved by the Animal Experimentation Ethics Committee of Ondokuz Mayıs University. All animals were individually caged in a room under standard environmental conditions and were fed with a standard rat diet.

Experimental groups

The offsprings of five Spraque-Dawley pregnant rats (body weight, 250–300 g) were assigned to individual experimental groups as described in Table 1. The mothers of the control group (CG) received no treatment and gave birth to seven offsprings. In the Sham group (SG), the mothers received 100 mg of nitrofen dissolved in 1 ml of olive oil by oral gavage on gestational day 9 and gave birth to seven offsprings. The mothers in the Group TRH that received 100 mg of nitrofen dissolved in 1 ml of olive oil by oral gavage on gestational day 9, and intraperitoneal (i.p.) injection of 25 µg/kg of TRH on gestational day 19 gave birth to eight offsprings. The mothers of the glucocorticoid group (GG) that received 100 mg of nitrofen dissolved in 1 ml of olive oil by oral gavage on gestational day 9, followed by 0.25 mg/kg of dexamethasone (i.p.) on gestational day 19 gave birth to 10 offsprings. The mother rats of the insulin group (IG) that received 100 mg of nitrofen dissolved in 1 ml of olive oil by oral gavage on gestational day 9 and 0.5 IU/kg of long-acting insulin on gestational day 19 (i.p.) gave birth to nine

Table 1. Design of experimental groups (CG, control group; SG, sham group; TRHG, thyrotropin-releasing hormone group; GG, glucocorticoid group; IG, insulin group).

Groups	Procedure Applied to the Pregnant Rat	The Number of the Offsprings (n)
CG	-	7
SG	nitrofen	7
TRHG	nitrofen + TRH	8
GG	nitrofen + dexamethasone	10
IG	nitrofen + insulin	9

Table 2. Modified APGAR scores for rats.

APGAR score	Skin color	Respiratory pattern	Muscular tonus
0	Blue	Absent	Akinesia and rigidity
1	Pink-blue	Gasping	Motion restricted to front legs and head
2	Pink	Vocalisation	Motion in whole body

offsprings. Newborn rats were delivered by cesarean section on 21st gestational day in order to standardise the APGAR score of the newborn rats, and to precisely evaluate the impact of different variables on APGAR scores.

Apgar scores

The modified Apgar score for each newborn rat was calculated by assessing the skin colour of (blue, pink-blue, pink), respiratory pattern (absent, gasping, vocalisation) and muscular tonus (akinesia and rigidity, motion restricted to front legs and head, motion in whole body) of the offsprings on a scale of 0 to 2 (Table 2) [11].

Diaphragmatic defect and the lung weight

All offsprings were sacrificed and underwent bilateral anterior thoracotomy and median sternotomy to observe defects on the diaphragm. The lung tissues were weighed.

Histopathological evaluation

The tissue samples were placed in 10% formalin solution, stained with haematoxylin and eosin dye, and then examined under a light microscope (HMLB45; Leica Microsystems, Wetzlar, Germany). The diameters of the alveoli and bronchi were measured and the number of alveoli was counted five times along a perpendicular line drawn from the centre of the respiratory bronchiole to the closest septum of the connective tissue, and then the mean values were calculated for analysis.

Statistical analysis

All statistical analyses were conducted with IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA). The normality of data was assessed using the Shapiro-Wilk test. The APGAR scores among groups were analysed by Kruskal Wallis test. The lung weights and number of bronchi and alveoli among groups were analysed by one-way analysis of variance. The Tukey HSD test was used to identify differences among the groups. The results are presented as median (min-max) or mean (± standard error of mean) depending on the distribution of the data. A p value of < 0.05 was considered as statistically significant.

Results

The mean Apgar scores of groups are given in Figure 1. Apgar scores were not significantly different among the Groups CG Group, TRHG and IG. Apgar scores of Groups CG and IG were significantly higher than the Group SG ($p < 0.001$). DH was observed in 0 (0.0%), 5 (71.4%), 1 (12.5%), 3 (30%) and 2 (22.2%) rats in the Groups CG, SG, TRHG, GG and IG, respectively. The incidence of diaphragmatic hernia was significantly different among the groups ($p=0.031$). Although the mean weight of the lung tissue was significantly different among the groups ($p<0.001$) (Figure 2), it was the highest in the Group

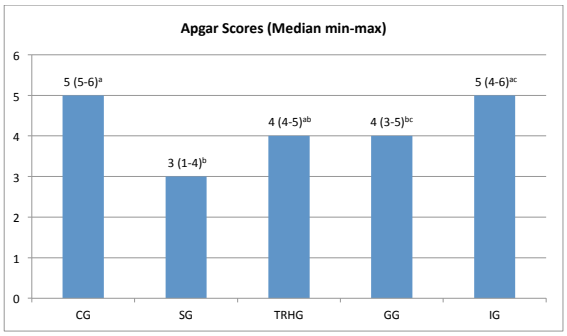

Figure 1. Comparisons of mean Apgar scores among groups (CG, control group; SG, sham group; TRHG, thyrotropin-releasing hormone group; GG, glucocorticoid group; IG, insulin group).
a,b,c Values marked with the same letter indicate that no differences exist between the groups (p<0.001).

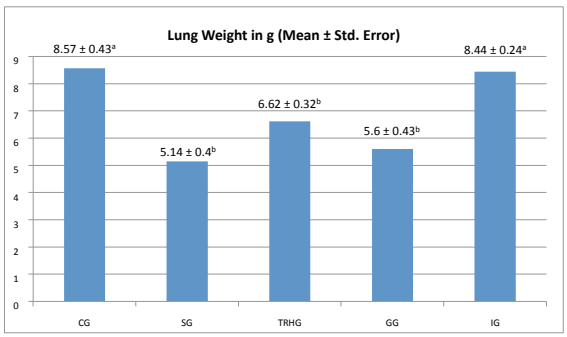

Figure 2. Mean lung weights in gram among groups (CG, control group; SG, sham group; TRHG, thyrotropin-releasing hormone group; GG, glucocorticoid group; IG, insulin group).
a,b Values marked with the same letter indicate that no differences exist between the groups (p<0.001).

CG, followed very closely by Group IG. The mean number of alveoli and bronchi were not different among the groups (p=0.410) (Figure 3).

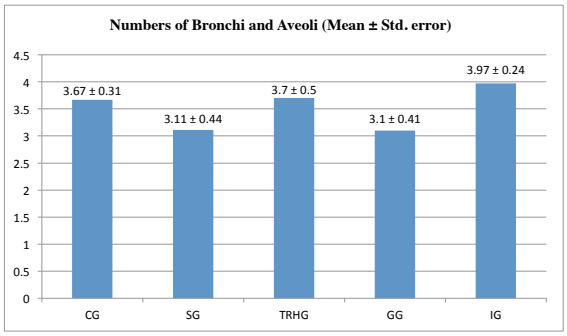

Figure 3. Mean numbers of bronchi and alveoli among groups (CG, control group; SG, sham group; TRHG, thyrotropin-releasing hormone group; GG, glucocorticoid group; IG, insulin group) (p=0.410).

Discussion

CDH is an anomaly with a high mortality rate and currently most patients are recognized in the antenatal period. The major cause of mortality in patients with CDH is PH [12]. A number of studies have investigated perinatal measures to prevent or alleviate the development of PH in CDH, such as administration of various pharmacological agents to mothers to promote maturation of the fetal lung [13-15]. Such agents, including thyroid hormones, corticosteroids and various vitamin derivatives are able to cross the placenta [16-18].

TRH, glucocorticoids and insulin are recognised as endocrine factors that influence fetal development both directly and indirectly. Although several studies have evaluated the effects of TRH and glucocorticoids for the treatment of nitrofen-induced diaphragmatic hernia, our study is the first that evaluated insulin for the treatment of CDH. The results of this study showed that insulin, in addition to TRH and glucocorticoid, has no detrimental effect on the development of fetal lung.

Nitrofen is the most commonly used agent for experimentally induced CDH in pregnant rats when given as oral gavage, but these studies reported varying incidence rates of diaphragmatic defects in the offsprings, ranging from 40% to 80% [19-21]. In our study its incidence was 71.4% in the Sham Group and comparatively lower in all treatment groups, with the lowest rate observed in the Group TRHG. Manson et al. argued that the toxic effects of nitrofen are mediated via thyroid hormones [7]. In contrast, TRH administration had significantly reduced the incidence of diaphragmatic defects in our study although this reduction may also be secondary to the possible alleviating impact of TRH on the effects of nitrofen. On the other hand, another report argued that the effect of nitrofen was independent of thyroid hormones [21] and the combined use with dexamethasone was found to promote the maturation of fetal lung [22]. In our study, although the Apgar scores in the Groups TRHG and CG were comparable, there were no significant intergroup differences in mean lung weight or the mean number of bronchi and alveoli. In terms of improvement in diaphragmatic hernia, Losty et al. observed no favourable changes with TRH treatment alone in any of the parameters in their study, but suggested that the combined use of a glucocorticoid and TRH yielded more significant results [23]. As it is unclear whether the underlying mechanism of nitrofen activity occurs via the thyroid hormones or not, the impact of TRH treatment should be investigated in CHD models induced by agents other than nitrofen and then impact of TRH should be evaluated, either alone or in combined treatment.

Taira et al. reported that following administration of nitrofen to pregnant rats on gestational day 9, and additional administration of dexamethasone on gestational day 19 promoted fetal lung development [24]. Maternal administration of glucocorticoids promotes structural and biochemical alterations of type 1 and type 2 pneumocytes and improves both lung mechanics and gas exchange. Furthermore, glucocorticoids improve surfactant production and absorption of alveolar fluid by stimulating pulmonary beta receptors [25,26].

However, in our study administration of dexamethasone produced no significant differences in Apgar scores, the incidence of diaphragmatic defects, lung weights and the mean number of alveoli and bronchi when compared with the untreated group. Similar to our findings, Merrill et al. reported that antenatal administration of a corticosteroid for CDH demonstrated no positive

effect on lung maturation [27]. Although there are conflicting opinions in the literature regarding the use of dexamethasone for CDH, some authors reported that the use of dexamethasone in combination with TRH might promote the development of fetal lung [22,23]. Ansari et al. reported that fetal lungs exposed to a higher dose of TRH had increased numbers of alveoli, air-blood barriers and lamellar bodies per type II cell [28].

In our study, the most remarkable results were observed in the Group IG. In rats with experimentally induced diabetes, lung maturation was delayed, alveolar development was encumbered, epithelial differentiation remained incomplete and lamellar bodies had accumulated type II pneumocytes [29]. Furthermore, maternal control of diabetes have shown to lead a normal course of fetal lung maturation [30]. In our study, mean Apgar scores were similar between the Groups IG and CG, and the incidence of diaphragmatic defects was lower in the Group IG. Although there were no statistically significant differences in the number of alveoli and bronchi between groups, the mean lung weight was lower in the nitrofen administered rats and slightly greater in the groups treated with TRH and glucocorticoid, as compared with the Group CG. However, lung weight was significantly greater in the Group IG and also the closest to normal lung.

The findings of our study may promote new understanding about the pathophysiological process of the CDH and thus may lead to new investigations to prevent and treat a significantly morbid and mortal condition. In our opinion, maternal administration of TRH should be studied in another experimental model. Although the results obtained by administering dexamethasone alone were not significant, it should be considered to be used in combination with other agents such as insulin, which had significantly favourable effects on Apgar scores, lung weight and the numbers of alveoli and bronchi. The results of the present study are expected to promote launching of new studies on the use of insulin therapy alone or in combination for antenatal treatment of CDH.

Ethics Committee Approval: Approval of Ondokuz Mayıs University Animal Experiments Local Ethics Committee was obtained (3.10.2016 / 178).

Conflict of Interest: The authors declare that they have no competing interest.

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