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Effects of lactational cyclosporine A use on rat pups

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Abstract: We aimed to evaluate the structural and functional changes in the thymus and kidneys of rat pups whose mothers were given cyclosporine A (CsA) during lactational period. Six adult nursing Wistar rats and their 30 pups were studied. Rat pups were divided into four groups as follows: 21-day treated group and 21-day placebo group, each including 10 breastfeeding pups sacrificed on the 21st day, whose mothers were given CsA or placebo, respectively (*infancy groups*) and, 60-day treated group and 60-day placebo group, each including five breastfeeding pups sacrificed on the 60th day, whose mothers were given CsA or placebo, respectively (*puberty groups*). While CsA levels of mother rats were very high, CsA levels of 21-day treated group pups were zero. There were no renal histomorphometric differences between study and control pups in both age groups. Renal function parameters showed significant differences between study and control pups in the infancy group: the 21-day treated group pups had significantly lower urine volume, proteinuria, FE_{Na} and urinary NAG/creatinine ratio. GFR was also lower in the 21-day treated group, but the difference was not significant, and serum creatinine levels were also not different. Renal function differences were not present among the pubertal pups. Thymic corticomedullary ratio of the 21-day treated group was significantly higher than the 21-day placebo group, while there was no difference between the 60-day treated group and 60-day placebo group. There were no significant changes in the number and distribution of CD3+, CD4+, and CD8+ thymocytes between study and control pups in both age groups. In conclusion, breastfeeding by CsA-treated mother rats induced structural alterations in the thymus and functional changes in the kidneys of the rat pups during infancy. Disturbances in the kidneys and thymus mostly improved after CsA exposure was over.

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When cyclosporine A (CsA) is given to pregnant rats, it damages the kidneys and delays the development of hemopoietic organs including the thymus in the newborn rats (1, 2). Pups that were exposed antenatally to CsA presented first a permanent nephron deficit, second, glomerular, tubular and intrarenal hemodynamic dysfunction, third, enlarged kidneys with numerous tubular and glomerular lesions, and, fourth, an endothelin-dependent systemic hypertension that worsened with age (3). A recent meta-analysis of clinical studies suggested that CsA could be associated with premature birth, but did not appear to be a major human teratogen (4). On the other hand, clinical data related to the immunological status of infants exposed to CsA *in utero* are controversial (5, 6).

The American Academy of Pediatrics cautions against breastfeeding because of the documented presence of cyclosporine in breast milk and unknown long-term effects on nursing infants

Abreviations: CsA, cyclosporine A; FE_{Na}, fractional sodium excretion; GFR, glomerular filtration rate; GSD, glomerular surface density; H&E, hematoxylin & eosin; MT, mason's trichrome; NAG, *N*-acetyl-β-D-glucosaminidase; NGS, number of glomeruli in the stroma; TRP, tubular reabsorption of phosphorus; $U_{NAG/Cr}$, urinary NAG/creatinine ratio.

(7). Breastfed infants of mothers treated with CsA had no demonstrable nephrotoxic effects or any other side effect, and, thus, women having kidney transplants could be allowed to breastfeed (8, 9). However, there is no available experimental study evaluating the effects of postnatal exposure to CsA through breast milk on the renal and hemopoietic tissues.

CsA given during lactation passes to breast milk and could effect the renal and thymic tissues of the rat pups both functionally and histopathologically in early and late postnatal life. The aim of this study was to evaluate both the structural and functional renal changes, and structural thymic changes in the breastfed rat pups whose mothers were given CsA throughout lactation.

Materials and methods

The study was approved by the Dokuz Eylül University Medical Faculty Ethical Committee.

Animals and experimental design

Six adult nursing Wistar rats weighing about 230 g and their pups were studied. The animals were housed in stainless-steel mesh cages placed in an environmentally controlled room (21°C, relative humidity at 55% and a day/night cycle of 12 h). They were fed standard rat pellets and municipial water *ad libitum*.

Mother rats were randomly allocated to two groups, each having three animals. as CsA-treated group and placebo-treated groups. CsA (Sandimmun, Novartis, Basel, Switzerland) was injected daily at a dose of 15 mg/kg of body weight subcutaneously for 21 consecutive days in the CsA-treated group, and isotonic saline was given by the same route and of the same volume in the placebo-treated group.

Rat pups were divided into four groups as follows: 21-day treated group and 21-day placebo group, each group having 10 breastfeeding pups sacrificed at the end of the lactation (21st day), whose mothers were given CsA or placebo, respectively (infancy groups). Sixty-day treated group and 60-day placebo group, each group having five breastfeeding rat pups sacrificed on the 60th day, whose mothers were given CsA or placebo, respectively (adolescence groups). All the rat pups were breastfed for 21 days and weighed at the end of the study.

Blood CsA level measurements

CsA concentrations were measured by the enzyme multi-immunoassay technique (EMIT; Behring, Cupertino, CA, USA) in the whole blood of mothers 12 h after the last dose and in rat pups at random hours in relation to the feeding at the end of the lactational period. The detection limit was 30 ng/mL.

Renal functions

Blood samples were obtained from tail veins of the rat pups. In addition, timed urine samples of the pups in metabolic cages were collected for 24 h.

Blood creatinine and phosphorus levels were measured using Sigma commercial kits (Procedure No: 557 and 360 UV, respectively, Sigma, Germany) by spectrophotometric methods, while the sodium level was measured by using the ion selective electrode method (OpeRA, Bayer, Germany, USA). Control sera (Sigmanorm and Sigmapath) were included in each run.

Urinary protein was assessed using the Biocon commercial kit (Cat No. 915 at 600 nm as pyrogallol red method, Biocon, India). Urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity was measured by using Boehringer Mannhaim commercial kit (Cat No. 875406, Roche, Germany), which included 3-cresosulfonphthaleinyl-NAG as substrate. The purple complex was measured spectrophotometrically (Jasco V550, Jasco, Tokyo, Japan) at 580 nm.

Glomerular filtration rate (GFR) as endogenous creatinine clearence, fractional sodium excretion (FE_{Na}), tubular reabsorption of phosphorus (TRP), proteinuria and urinary NAG/creatinine ratio ($U_{NAG/Cr}$) were evaluated for each rat pup.

Pathology

Kidney tissues were fixed in formalin and 3 μm sections were stained by hematoxylen & Eosin (H&E), modified Masson's trichrome (MT), periodic acid Schiff (PAS) and periodic acid methanamine silver (PAMS) stains. Half of the thymic tissues were fixed in formalin, while frozen sections were performed in the remaining non-fixed tissues.

Morphometric evaluation of the kidney

Interstitial fibrosis, and vascular and tubular structures of the kidneys were determined by the morphometric point counting method in sections stained by MT. A microscopic image, obtained with a ×10 objective, was projected by a CCD camera (Sony, Japan) to a monitor (Sony) attached to the microscope (Nikon, Optiphot, Japan). The representative field on the monitor was superimposed with a transparency containing the counting frame, with 11 horizontal and vertical lines making up 121 points of intersection. A total number of 1815 points falling on the tubulus, fibrotic interstitial tissue, glomeruli and vascular structures of the cortical area at 15 fields were counted (10–12).

The percentage of sclerotic glomeruli was determined by dividing the number of sclerotic glomeruli to number of total glomeruli.

In H&E-stained renal tissue sections, glomerular surface density (GSD) and the number of glomeruli in the stroma (NGS) were assessed stereologically, at ×84 final magnification. A Thoma counting chamber (Heinz Herenz, Hamburg, Germany) was used to determine the distances between the lines on the monitor. GSD and NGS were calculated as follows: GSD in $\mu m^{-1} = (\sum I_n.2.121)/I_{STR}.L_R)$ and NGS in $\mu m^{-2} = (N.121)/I_{STR}$, where L_R is the distance between the lines on the slide, I_{STR} is the number of intersection points outside the glomeruli, I_n is the number of intersections within the glomeruli and N is the number of glomeruli in the measurement field (9–11).

Measurement of afferent arterioles

The images obtained from the microscope were transferred by a Hitachi video camera (Model WK-C220E, Hitachi Ltd., Japan) to a computer. The images were captured and analyzed by Bs200Doc Module (BAB Bilgisayar Mühendislik Ltd., Republic of Turkey). The images were calibrated for ×40 microscopic magnification and measurements were performed as micrometers. The distance between the two luminal borders of the afferent arteriol just at the level of

Bowman's capsule was measured with a point interactive method through a vector. Ten glomeruli were measured for each case after mapping the glomerular profiles for choosing the section with maximum vectorial distance (10–12).

Morphometric evaluation of the thymus

A similar point counting method described for the morphometric evaluation of the kidneys was applied. Points falling on the medulla and the cortex of the thymus were counted in 10 fields in H&E-stained sections with ×4 objective (10–12).

Immunohistochemistry

Frozen sections from thymus tissues and sections from formalin-fixed thymus tissue blocks were placed on poly-I-lysine slides. The avidin–biotin–peroxidase method was performed using the primary antibodies against CD3, CD4 and CD8 (Prediluted, Dako Corp, Glostrup, Denmark). Briefly after deparaffinization of paraffin embedded tissues 0.3% solution of hydrogen peroxidase was applied for blocking endogenous peroxidase activity for both frozen and paraffin-embedded sections. Then they were washed in phosphate-buffered saline (PBS). Primary antibodies were applied at room temperature for 30 min. Biotinilated secondary antibody and streptavidine peroxidase complex were added consecutively for 10 min at room temperature and washed in PBS. The peroxidase activity was visualized with 3-3′-diaminobenzidine tetrahydrochloride (DAB) applied for 5 min. Finally, sections were counterstained by hematoxylin, dehydrated and mounted. Sections from tonsillectomy specimens were labelled as positive controls for each primary antibody. Also, negative controls were performed. Evaluation of immunohistochemical stains; the distrubition of CD3+, CD4+ and CD8+ cells was scored semi-quantitatively for the medulla and cortex of the thymus.

Results were expressed as mean ± standard deviation. Statistical evaluation of the results was performed by Mann–Whitney *U*-test, in which p less than 0.05 was accepted as significant.

Results

Mean blood CsA level of the mother rats was 1773 ± 1059 ng/mL. On the other hand, it was zero in pups of these rats, except one with a blood CsA level of 32 ng/mL.

Mean weights of the pups were significantly lower in 21-day treated group than in 21-day placebo group (32.4 ± 2.4 vs. 46.8 ± 13.0 g, $p = 0.002$), but were not different in 60-day treated group and 60-day placebo group (207.4 ± 62.2 vs. 184.6 ± 11.2 g, $p = 0.420$).

When we compared the renal functional parameters of the infancy groups, urine volume, proteinuria, FE_{Na} and $U_{NAG/Cr}$ ratio were significantly lower, and TRP was significantly higher in the pups of CsA-treated mothers (21-day treated group). On the other hand, serum creatinine and GFR, although lower in the 21-day treated group, were not significantly different (Table 1).

Comparison of the renal functional parameters between the adolescence groups revealed no significant difference except TRP, which was significantly lower in the pups of CsA-treated mothers (60-day treated group) (Table 1).

By light microscopic examination, there were no remarkable changes in any group considering the glomeruli, interstitium, tubules and vascular structures of the renal tissues.

Histomorphometric evaluation of kidney tissue demonstrated that afferent arteriole diameter was significantly smaller in the 21-day treated group compared with the 21-day placebo group. Other parameters were not different (Table 2).

Renal histomorphometric parameters including afferent arteriole diameter*s* were not different between the adolescence groups (Table 2).

Histomorphometric analysis of the thymus showed that corticomedullary ratio increased significantly in the 21-day treated group compared with the 21-day placebo group (2.6 ± 0.5 vs. 1.4 ± 0.09, $p = 0.001$). Adolescence groups were not different (0.99 ± 0.09 vs. 1.00 ± 0.11 in the 60-day treated group and 60-day placebo group, respectively, $p = 1.000$). In Fig. 1, histopathological sections of the thymic tissues of the rat pups are seen. On the left is the thymic tissue of a pup in the 21-day treated group demonstrating a decreased ratio of medullary structures. On the right is the thymic tissue of a pup from the 21-day placebo group. Corticomedullary ratio was similar in the 60-day treated group and the 60-day placebo group (Fig. 2). On the other hand, corticomedullary ratio *was* significantly decreased at adolescence in both the CsA-treated and placebo-treated rat pups (2.6 ± 0.5 vs. 0.99 ± 0.09 in the 21-day treated group and the 60-day treated group, $p = 0.003$; and 1.4 ± 0.09 vs. 1.0 ± 0.11 in the 21-day placebo group and the 60-day placebo group, $p = 0.003$).

Semi-quantitative scores of CD3+, CD4+ and CD8+ cells were similar in both age groups and in both the study and control rat pups ($p > 0.05$).

Discussion

Pups that were exposed antenatally to CsA presented first a permanent nephron deficit, second, glomerular, tubular, and intrarenal hemodynamic dysfunction, third, enlarged kidneys with numerous tubular and glomerular lesions, and fourth, an endothelin-dependent systemic hypertension that worsened with age (3). Our study was not a model of renal transplantation. Rat pups had no exposure to cyclosporine during their fetal life. In this study, only the effect of cyclosporine intake via breast milk on rat pups during infancy was evaluated.

Table 1. Comparison of the renal functions between the groups

	21-day treated group	21-day placebo group	p	60-day treated group	60-day placebo group	p
Serum creatinine (mg/dL)	0.49 ± 0.15	0.57 ± 0.10	0.190	0.56 ± 0.16	0.40 ± 0.10	0.151
Urine volume (μL/kg/day)	14481 ± 12440	52625 ± 50035	0.008	32670 ± 8234	25681 ± 4595	0.151
GFR (μL/kg/min)	1252 ± 833	1619 ± 731	0.579	4365 ± 1343	5719 ± 971	0.061
Proteinuria (mg/kg/day)	46.0 ± 8.2	338.9 ± 149.3	0.001	229.6 ± 108.2	195.1 ± 51.4	0.548
TRP (%)	99.62 ± 0.004	99.22 ± 0.004	0.025	93.18 ± 0.004	99.40 ± 0.003	0.008
$U_{NAG/Cr}$ (mU/mg)	8.94 ± 5.51	19.53 ± 9.10	0.019	18.29 ± 4.75	12.81 ± 6.57	0.222
FE_{Na} (%)	0.98 ± 0.09	2.11 ± 0.83	0.019	0.45 ± 0.09	0.62 ± 0.17	0.950

Values are expressed as mean ± standard deviation.
GFR, glomerular filtration rate (as endogenous creatinine clearance); TRP, tubular reabsorption of phosphate; UNAG/Cr, Urinary *N*-acetyl-β-D-glucosaminidase/creatinine ratio; FENa, fractional excretion of sodium.

Table 2. Comparison of the renal histomorphometric parameters between the groups

	21-day treated group	21-day placebo group	p	60-day treated group	60-day placebo group	p
Afferent arteriole diameter (μm)	4.39 ± 0.55	5.68 ± 0.42	<0.001	8.82 ± 0.49	8.31 ± 0.67	0.222
Glomerulus (%)	6.93 ± 0.62	6.67 ± 0.87	0.460	7.12 ± 1.74	6.16 ± 1.94	0.540
Tubulus (%)	91.21 ± 0.82	91.37 ± 0.96	0.760	93.26 ± 3.29	92.12 ± 2.15	0.840
Vessels (%)	0.95 ± 0.21	0.93 ± 0.23	1.000	0.88 ± 0.43	1.26 ± 0.65	0.420
Interstitium (%)	0.97 ± 0.40	1.01 ± 0.28	0.830	0.74 ± 0.32	0.46 ± 0.11	0.150
NGS (μm^{-2})	6.67 ± 1.62	6.50 ± 1.51	0.890	3.59 ± 1.07	3.79 ± 0.26	0.690
GSD (μm^{-1})	26.02 ± 5.09	28.75 ± 6.75	0.360	25.01 ± 8.57	24.92 ± 1.22	0.690

Values are expressed as mean ± standard deviation.
NGS, number of glomeruli in the stroma; GSD, glomerular surface density.

There was no experimental study investigating the effects of lactational CsA use on rat pups in detail at the onset of our study. More recently, a paper investigating T-cell maturation and function in rat pups breastfed by CsA-treated mothers was published (13). The major limitation to the use of CsA is nephrotoxicity (14). It was shown previously that infants of mothers treated with CsA had normal serum creatinine levels and body weights during lactation, but it was not possible to follow-up structural changes of renal and thymic tissues in these cases (8, 9). In the present study, the effects of lactational CsA on structural and functional changes of renal and thymic tissues in rodents during developmental periods were evaluated.

The main adverse reaction to the immunosuppressive drug cyclosporine is dose-dependent renal dysfunction. Renal vasoconstriction without major tubular dysfunction is usually noted (15). CsA exposure during pregnancy and/or during breastfeeding does not induce neprotoxic

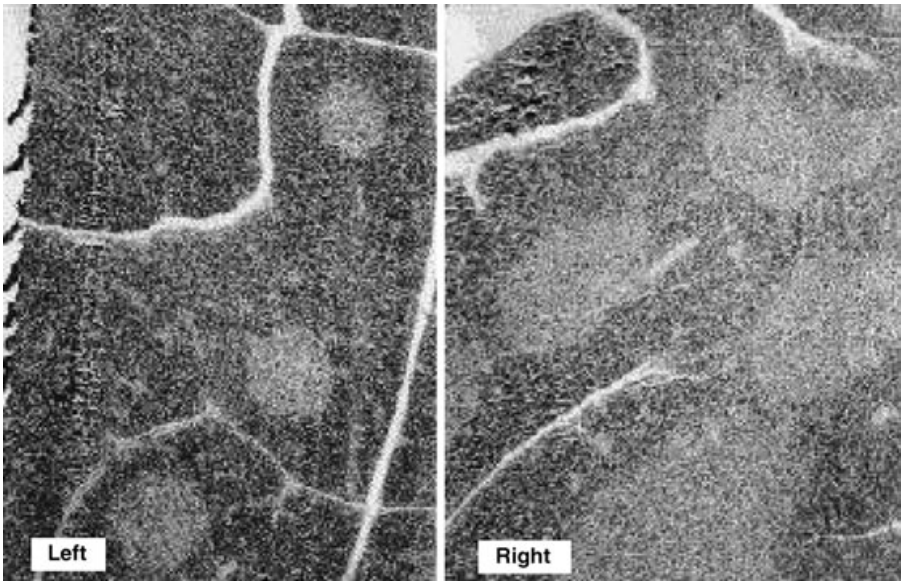

Fig. 1. Histopathological sections of the thymic tissues of the rat pups. On the left is the thymic tissue of a rat pup of a cyclosporine A (CsA)-treated mother sacrificed at the end of the lactational period demonstrating decreased ratio of medullary structures. On the right is the thymic tissue of a rat pup of a non-CsA-treated mother sacrificed at the end of the lactational period.

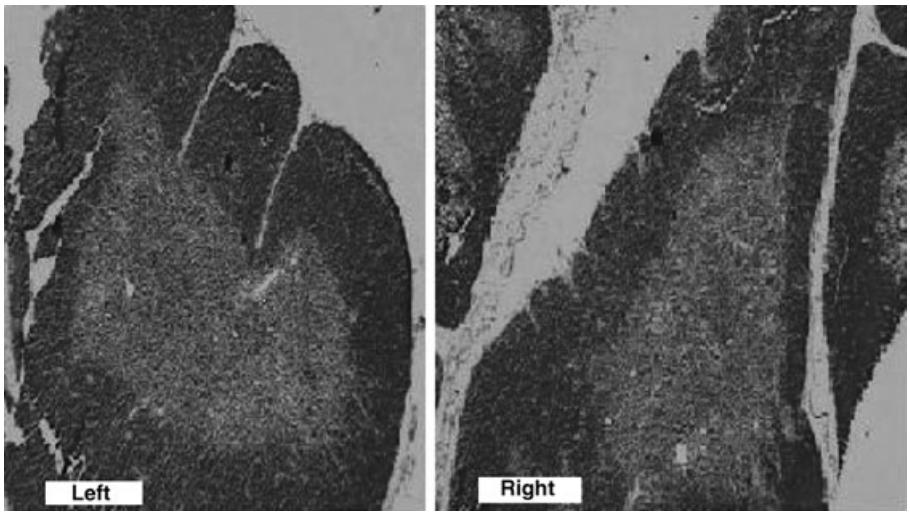

Fig. 2. Histopathological sections of the thymic tissues showed similar cortical and medullary structures in the adolescence groups. On the left is the thymic tissue of a rat pup of a cyclosporine A (CsA)-treated mother sacrificed on the 60th day. On the right is the thymic tissue of a rat pup of a non-CsA-treated mother sacrificed on the 60th day.

effects in the fetus or infant (8, 16). Lower urine volume, GFR, proteinuria and FE_{Na} during infancy in the rat pups of CsA-treated mothers could be because of the afferent arteriolar vasoconstrictive effect of CsA (17, 18). The afferent arteriolar vasoconstriction rather than direct tubular injury is a major pathogenic factor in experimental CsA nephrotoxicity (19). In the 60-day treated group, renal function other than TRP normalized following discontinuation of CsA.

Bennnett et al. (20) have shown that there was no evidence of tubuler necrosis by light or electron microscopy despite the depression of renal hemodynamics during CsA treatment. Duncan et al. (21) have shown that use of the electron microscope improves detection of early and minimal tubular cell damage, ultrastructural changes because of CsA intake are evident although the renal tubules are apparently unaffected when examined by light microscopy. CsA is reported to induce tubular epithelial damage whereas histological examination of the glomeruli does not reveal abnormalities unless particularly high doses of the drug are used (22). In this study, remarkable renal histomorphologic changes were not found by light microscopic examination in any group.

On the other hand, lower serum creatinine level in the 21-day treated group could be because of lower body mass of this group of rats related to anorexia and increased catabolism caused by CsA (23). It was reported that high dose CsA can lead to actual weight loss or failure to gain weight (13). The CsA levels in the mothers were extraordinarily higher than might be expected clinically. Maternal anorexia because of high blood CsA levels could impact maternal milk production and therefore oral intake of fluids. Undetectable hypovolemia of rat pups could cause reduced urine output and fractional excretion of sodium. The experimental feeding of breast milk to both groups would have been ideal to compare the breast milk intake of rat pups between groups. We avoided the handling of rat pups because of maternal cannibalism.

Neonatal exposure to CsA via lactational transfer can cause significant alterations in T-cell maturation and inhibition of lymphoproliferative responsiveness to mitogen activation (13). In this study, following CsA exposure the medulla disappeared in the tissue sections of the thymus. Thirty days after discontinuation of CsA, restored medullary architecture was observed. Conventional histopathological techniques have been reported to be sufficient to identify potential immunotoxicants without the need for immune function tests (24). Thus, although we evaluated only the histopathological alterations in the thymic tissues, the results helped us to evaluate the thymic function of the pups. As expected, corticomedullary ratio increased during exposure to CsA, during the lactational period, in rat pups of the 21-day treated group (25, 26). Furthermore, after CsA exposure was over, normal thymic evolution associated with age was preserved in this group of exposed rat pups as in controls.

However, the number and distribution of CD3+, CD4+ and CD8+ cells were similar in the 21-day treated group and the 21-day placebo group. The same observation was made experimentally in the rats exposed to CsA *in utero* (5). The reduced percentage of CD4+ mature thymocytes in rat pups of CsA treated mothers was reported (13). It may be because of changes in the sensitivity of rat strains to CsA or different CsA levels in rat pups.

Human studies have shown CsA concentrations in breast milk to be as high as 440 ng/mL but below detectable blood levels (8). At the 15 mg/kg/day dose, the neonatal CsA blood concentration was significantly less (−55%) than the maternal CsA blood concentration after 20 days of maternal CsA treatment (13). The discrepancy may be because of sampling time or different intestinal absorptive characteristics of rats. CsA produces detectable effects at the lowest dose on the immune system shown by histopathological techniques and immune function tests (24). The observed toxic effects of CsA in rat pups of the 21-day treated group in spite of unmeasurable blood levels of the drug might be because of either unmeasured metabolic compounds of CsA with biological activity (27) or the effect of even these low CsA levels on these young animals. Babies are thought to receive a large dose of cyclosporine because blood and milk concentrations are similar, but this is not so and is just one of many pharmcological factors. Although fetusus may be exposed to blood cyclosporine concentrations that are about one-third of maternal amounts, no adverse effects have been described; risks from the much lower quantities during breastfeeding are likely to be minimal (28, 29). Human milk confers major benefits; advice on breastfeeding should balance the measured risk from maternal drugs with the disadvantages of formula feeding.

In analyzing outcomes in female liver recipients, no specific graft or newborn outcome differences have been noted when a comparison has been made between different calcineurin inhibitor regimens (30).

In conclusion, breastfeeding by CsA-treated mother rats induced structural alterations in the thymus and functional changes in the kidneys of the rat pups during infancy. Disturbances in the kidneys and thymus mostly improved after CsA exposure was over and permanent damage was not found in these organs. Pros and cons of breastfeeding in nursing mothers during CsA treatment should be balanced.

Acknowledgments

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