Acta Medica Alanya

RESEARCH ARTICLE

ARAŞTIRMA

Acta Medica Alanya

2021;5(1): 4-10

DOI:10.30565/medalanya.775667

Determination of effects of chemical agents on liver fibrosis models frequently used in different doses and time periods

Karaciğer Fibrozis Modellerinde Sık Kullanılan Kimyasal Ajanların Farklı Doz ve Zaman Dilimindeki Etkilerinin Belirlenmesi

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ABSTRACT

Aim: In this study, it was aimed to reveal a more effective model depending on the dose and time by evaluating histopathological properties and biochemical parameters, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, triglyceride, cholesterol in carbon tetrachloride and thioacetamide (CCl_4 and TAA) models.

Method: Rats were divided into three groups for each model and intraperitoneally (i.p.) injected with CCl_4 (0.5 ml/kg, 1.0 ml/kg, 2.0 ml/kg) and TAA (100 mg/kg, 200 mg/kg, 300 mg/kg) for 4, 6 and 8 weeks, three times weekly, respectively.

Results: In the biochemical investigation, ALT and AST values in the only 0,5 ml CCL4 of groups for 6 and 8 weeks and were found to have significant differences compared to the control groups ($p < 0.05$), while the other biochemicals parameters values did not reveal significant difference in the groups ($p > 0.05$). According to the results of the histopathology in the liver tissues, both the control groups showed a normal histological feature. The hepatofibrotic alterations were remarkable in the CCl_4 and TAA models fibrosis depending on the increasing dose and time in all of the groups.

Conclusion: Our results showed that the dose and time were reached up to until the cirrhosis for eighth week. These results would be a helpful reference for hepatofibrotic studies.

Keywords: TAA, CCl_4, Liver, Fibrosis

ÖZ

Amaç: Bu çalışmada, karbon tetraklorür ve tiyoasetamid (CCl_4 ve TAA) modellerinde alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), albümin, trigliserit ve kolesterol gibi biyokimyasal parametreler ve histopatolojik özellikler değerlendirilerek doz ve zamana bağlı olarak daha etkin modelin ortaya çıkarılması amaçlanmıştır.

Yöntem: Her bir model için ratlar 3 gruba ayrılmıştır ve intraperitoneal (i.p.) olarak CCl_4 (0.5 ml/kg, 1.0 ml/kg, 2.0 ml/kg) ve TAA (100 mg/kg, 200 mg/kg, 300 mg/kg) 4, 6 ve 8 hafta boyunca hafta da üç kez enjeksiyon yapılmıştır.

Bulgular: Biyokimyasal araştırmalar sonucunda ALT ve AST değerleri, sadece 0,5 ml CCL_4 6. ve 8. hafta gruplarında kontrol gruplarına göre istatistiksel olarak anlamlı fark göstermiştir ($p<0.05$). Diğer biyokimyasal parametrelerin değerleri kalan gruplar arasında anlamlı farklılık göstermemiştir ($p>0.05$). Histopatolojik sonuçlara bakıldığında karaciğer dokusunda, kontrol gruplarının her ikisinde de karaciğer, nor-mal histolojik yapısını göstermiştir. Diğer bütün gruplarda, artan zaman ve doza bağlı olarak her iki modelde göze çarpan hepatofibrotik değişiklikler gözlemlenmiştir.

Sonuç: Doz ve zamana bağlı olarak sekizinci haftaya ulaşan gruplarda siroz geliştiği gözlemlenmiştir. Bu sonuçlar hepatofibrotik çalışmalar için yarayışlı birer referans olabilecektir.

Anahtar Kelimeler: TAA, CCl4, Karaciğer, Fibrozis.

Received: 30.07.2020 Accepted: 27.11.2020 Published(Online): 23.04.2021

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To cited: Kaan D, Toprak G, Yay AH, Başkol G, Ertekin T, Ülger H. Determination of effects of chemical agents on liver fibrosis models frequently used in different doses and time periods. Acta Med. Alanya 2021;5(1):4-10. doi:10.30565/medalanya.775667

INTRODUCTION

Fibrosis is defined as excessive collagen regulation due to only minor clinical symptoms or new fiber formation that causes disruption in cell function [1]. Liver fibrogenesis is the ultimate common consequence of liver damage, a critical factor leading to liver dysfunction, and may be important in the pathogenesis of other chronic problems, portal hypertension [2] and biliary cirrhosis [3,4]. Hepatic fibrosis is a disease characterized by the accumulation of the extracellular matrix (ECM) following liver damage and can be treated by early diagnosis. If the damage to the liver is acute or with limited destruction, it can return to its normal structure. In case of ECM accumulation, it becomes permanent by replacing it with the parenchyma for wound treatment. This process results in cirrhosis in case of advanced fibrosis, with a high mortality rate [5]. Experimental animal models and cell culture methods will be helpful in understanding potential reversal of hepatic fibrosis and the mechanism underlying the activation of hepatic stellate cells [6]. CCL_4 and TAA are the most commonly used chemical agents in fibrotic studies, due to the fact that they are easy to apply and reproducible [7]. CCL_4 is metabolized by cytochrome P450 enzymes in the liver and converts to the highly reactive trichloromethyl (CCl_3), therefore it causes inflammation and fibrosis in the liver. The chronic application of CCl_4 has long been one of the most widely accepted models of acute-onchronic liver failure (ACLD), although it can also be used in shorter protocols for acute liver injury studies. In general, CCl_4 is administered to rats or mice through intraperitoneal injection or inhalation [8]. TAA is the second most-used model of hepatotoxin-induced ACLD after the CCl_4 and it has recently been used frequently, in both mice and rats. TAA is an organosulfur compound used in textile, paper, leather production and laboratories. It causes chronic liver damage as it affects protein synthesis, RNA, DNA and gamma glutamyl transpeptidase activity [9]. In this study, by inducing at the same time two different chemotoxins, CCl_4 and TAA, in animal models, it was possible to observe liver pathological features.

MATERIAL AND METHODS

Experimental animals

All animal procedures and experimental protocols were approved by the Experimental Animals Ethics Committee of Erciyes University, Turkey (12/89-12/08/2012). In total, 72 male Wistar-Albino rat of about 8 to 10 weeks of age, with an average body weight of 200 to 250 g, were procured from Laboratory Animal Unit of Experimental and Clinical Research Center of Erciyes University. They were held under controlled conditioning (25±1 °C constant temperature, 55% relative humidity, 12 h dark/ light cycles), while food and water were allowed ad libitum during the study period. The rats were acclimatized to laboratory conditions for 7 days before commencement of the experiment.

Treatment Schedule of Chemical Agencies

Properties of the Control Groups

Eighteen (18) rats were randomly divided into two groups as control groups.

Group 1: negative olive oil control group (n=9), 9 rats were randomly divided into three groups: Group 1.1. (n=3) olive oil was injected i.p. (intraperitoneal) three times a week for four weeks, Group 1.2. (n=3) olive oil was injected i.p. three times a week for six weeks and Group 1.3. (n=3) olive oil was injected i.p. three times a week for eight weeks.

Group 2: negative saline solution control group (n=9), 9 rats were randomly divided into three groups: Group 2.1. (n=3) saline solution was injected i.p. three times a week for four weeks, Group 2.2. (n=3) saline solution was injected i.p. three times a week for six week and Group 2.3. (n=3) saline solution was injected i.p. three times a week for eight weeks.

Properties of the CCL_4 Groups

Group 3: (n:9); 9 rats were randomly divided into three groups: Group 3.1. (n=3) 0.5 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for four weeks, Group 3.2. (n=3) 0,5 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for six weeks and Group 3.3. (n=3) 0,5 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for eight weeks.

Group 4: (n:9); 9 rats were randomly divided into three groups: Group 4.1. (n=3) 1 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for four weeks, Group 4.2. (n=3) 1 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for six weeks and Group 4.3. (n=3) 1 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for eight weeks.

Group 5: (n:9); 9 rats were randomly divided into three groups: Group 5.1. (n=3) 2 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for four weeks, Group 5.2. (n=3) 2 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for six weeks and Group 5.3. (n=3) 2 ml/kg CCl_4 in 20% olive oil was injected i.p. three times a week for eight weeks.

Properties of TAA Groups

Group 6: (n:9); 9 rats were randomly divided into three groups: Group 6.1. (n=3) 100 mg/kg TAA was injected i.p. three times a week for four weeks, Group 6.2. (n=3) 100 mg/kg TAA was injected i.p. three times a week for six weeks and Group 6.3. (n=3) 100 mg/kg TAA was injected i.p. three times a week for eight weeks.

Group 7: (n:9); 9 rats were randomly divided into three groups: Group 7.1. (n=3) 200 mg/kg TAA was injected i.p. three times a week for four weeks, Group 7.2. (n=3) 200 mg/kg TAA was injected i.p. three times a week for six weeks and Group 7.3. (n=3) 200 mg/kg TAA was injected i.p. three times a week for eight weeks.

Group 8: (n:9); 9 rats were randomly divided into three groups: Group 8.1. (n=3) 300 mg/kg TAA was injected i.p. three times a week for four weeks, Group 8.2. (n=3) 300 c mg/kg TAA was injected i.p. three times a week for six weeks and Group 8.3. (n=3) 300 mg/kg TAA was injected i.p. three times a week for eight week.

The blood samples were collected from the heart of every 3 animals at the end of the fourth, sixth, eighth weeks for all groups, which were then sacrificed. Blood samples were used for biochemical investigation and following sacrification, liver tissue was removed and examined for histological parameters.

Evaluation of Serum Biochemical Analysis

Serum was separated by centrifugation (3000xg, 15 min) following clotting of the blood. Serum AST, ALT, albumin, cholesterol and triglyceride levels were determined using a Cobas 8000 (Erciyes University)

Evaluation of Histopathological Parameters

Tissues samples were fixed in neutral 10% buffered formalin (pH 7.2) at room temperature. After fixation, tissues were dehydrated through graded alcohol solutions and embedded in paraffin. Sections (5 µm thickness) were stained with Masson trichrome and examined under a light microscope (Zeiss Axiolab) for histopathological analysis. The degree of fibrosis of liver sections was graded numerically based on the following criteria: 0, no fibrosis; I, slight fibrosis, fibrosis located in the central liver lobule; II, moderate fibrosis, widened central fibrosis; III, severe fibrosis, fibrosis extended to the edge of liver lobule; IV, liver cirrhosis.

Statistical Analysis: Compliance with the normal distribution of data and variance homogeneity were assessed by the Shapiro-Wilk and Levene test, respectively. Comparisons between groups were evaluated using the Kruskal-Wallis H tests and one way variance analysis. Multiple comparisons were evaluated using the Tamhan T2 and Siegel-Castell tests. Data analyses were evaluated using the IBM SPSS Statistics 20.0 commercial package programs (IBM Inc., Chicago, IL, USA) and significance level was assumed at roughly $p<0.05$, $p<0.001$.

RESULTS

Comparisons of Final Body

Throughout the experiments, the body weight of most treated animals decreased regularly. The animals started to die from the 4th week of treatment and continued to do so until the last day of cessation of observation. In total, 12 rats died during the whole observation period, 3 died after the cessation of 1 ml/kg CCl_4 at the end of 6th week and 6 died after the cessation of 2 ml/kg CCl_4 at the end of 4th week. Three died after the cessation of 300 mg/kg TAA at the end of 6th week. After the injection of chemical agents, the maximum weight loss was observed in group 5.1.

among the experimental groups. But the weight loss was found to be significantly decreased in group 3.2., group 4.1., group 6.2. and group 7.3 when compared with the control groups ($p<0.05$). In addition, the body weight changes were significantly different between CCl_4 and TAA model ($p<0.05$) (Table.1).

Table 1. The average weight changes in control and experiment groups

Groups	Initial weight (g) Mean ± SD	Weight after treatment(g) Mean ± SD	Body weight change (g)
1.1	223.6 ± 0.5	225.3 ± 0.5	+2
1.2	225.3 ± 1.5	227.0 ± 2.0	+2
1.3	225.6 ± 1.5	227.6 ± 1.5	+2
2.1.	222.3 ± 2.5	223.6 ± 2.0	+1
2.2.	222.0 ± 1.0	223. 6 ± 1.1	+1
2.3.	225.0 ± 1.0	226.6 ± 0.5	+1
3.1.	235.6 ± 2.0	232,3 ± 2.3	-3
3.2.	242.0 ± 2.0	237.3 ± 2.5	-5
3.3.	247.0 ± 1.0	241.0 ± 1.0	-6
4.1.	245.0 ± 1.0	240.3 ± 1.5	-5
4.2.	247.6 ± 0.5	241.0 ± 1.0	-6
5.1.	249.3 ± 0.5	241.0 ± 1.0	-8
6.1.	247.6 ± 0.5	244.0 ± 2.0	-3
6.2.	248.0 ± 1.0	243.0 ± 1.0	-5
6.3.	247.6 ± 0.5	241.0 ± 1.0	-6
7.1.	244.6 ± 0.5	241.3 ± 0.5	-3
7.2.	246.3 ± 0.5	243.0 ± 1.0	-3
7.3.	248.3 ± 0.5	243.6 ± 1.5	-5
8.1.	249.0 ± 1.0	245.6 ± 1.1	-4
8.2.	249.0 ± 1.0	242.3 ± 1.5	-7

Values are expressed as n (%) ($p<0.05$).

Comparisons of Serum Biochemistries

The serum albumin, cholesterol and triglyceride levels were increased by two chemotoxins injections including TAA and CCl_4 at all of the experimental groups, but this increase was not significantly compared with the control groups ($p>0.05$) and it was not significant among of the experimental groups either ($p>0.05$).

Both the serum ALT and AST were markedly increased at the end of the fourth week, after cessation of CCl_4. ALT and AST levels at group 3.2. that experimental group of CCl_4 model were significantly increased, compared with control groups ($p<0.05$). By the 6th week following cessation of TAA with group 6.3., the serum ALT was also markedly increased and this notable increase was found significantly different, compared with the control groups ($p<0.05$). AST levels at group 7.3. increased in comparison to the control groups but this increase was not found statistical significantly among them and the experimental groups ($p> 0.05$). AST levels at the groups 8.1 and 8.2 were increased compared to the control groups and this increase was found statistically significantly different ($p<0.05$). ALT levels at the other TAA groups except for group 7.3. were not found statistical significantly compared to the control groups. The level of serum ALT and AST are shown in Table. 2.

Table 2. Changes of serum ALT and AST levels and ± SD

Groups	n	AST (U/L)	ALT (U/L)
1.1.	3	88±1.5	42±1
1.2.	3	88±1	43±1
1.3.	3	92±2	70±1.5
2.1.	3	97±1.1	53±1.5
2.2.	3	97±2	66±0.5
2.3.	3	67±5.2	54±1.5
3.1.	3	102 ±9.5	75.6±7
3.2.	3	240± 138	210±133
3.3.	3	151±31	156±20.1
4.1.	3	131±7.6	117±3
4.2.	3	130±4.5	112±3.2
5.1.	3	211±3.2	142±37.4
6.1.	3	86±10.1	64±8
6.2.	3	121±10.2	67±9
6.3.	3	73±4.7	61±8.5
7.1.	3	88±4.5	63±12
7.2.	3	88±14.1	70±9.2
7.3.	3	146±43.5	86±8.5
8.1.	3	106±3.5	68±10.4
8.2.	3	129±34.7	78±10.4

Values are expressed as n (%) ($p<0.05$).

Histopathological Changes

The masson trichrome-stained histopathological appearance revealed normal hepatocytes morphology and intact hepatic lobules architecture in untreated control rats. According to the histopathological findings, liver tissue of normal control groups exhibited normal parenchymal structure features and normal architecture of hepatocytes radiating chord from the central vein (Figure 1).

CCl_4 intoxicated rats showed that collagen

deposition accumulates around the vena centralis, portal areas and blood vessels. Due to increased of CCL4 dose, the degree of fibrosis was increased as well. In addition to fibrosis, it was observed in notable necrosis and inflammation (Figure 2).

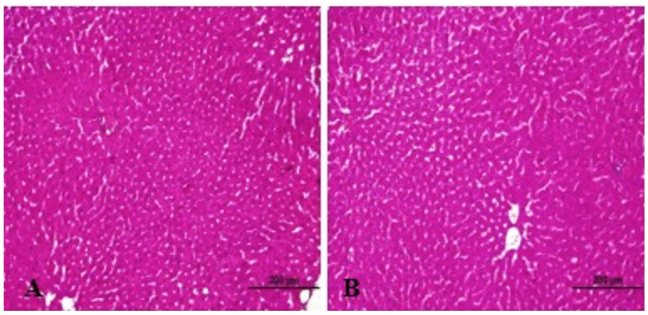

Figure 1: Section of liver obtained from control groups; group1 and 2: Group 1, no marked pathological changes A; group 2, no marked pathological changes and B; (×20).

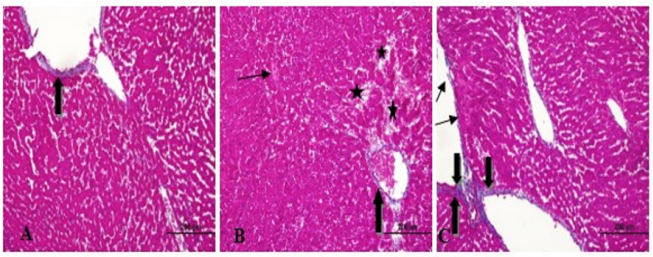

Figure 2: Liver histopathology of CCl_4-treated rats: Group 3.2. fibrosis (black thick arrows presented the fibrosis) tissue can be seen, it extended to the edge of liver lobule A; group 4.1. hemorrhagic necrosis (black thin arrows presented the necrosis) and inflammation (stars shapes presented the inflammation) B; group 5.1. necrosis, inflammation (black thin arrows presented the necrosis) and wide infiltration of inflammatory cells around the central veins (black thick arrows presented the fibrosis) C; (×20).

TAA intoxicated rats showed a higher degree of fibrosis and hepatic damage compared to the CCl_4 groups. Disruption of hepatic cell cord and infiltration of inflammatory cells were observed. Increased vacuolization and acidophilus bodies in the cytoplasm were also seen in the liver section.

The CCl_4 and TAA group showed notable bridging necrosis, inflammation and wide infiltration of inflammatory cells, around the central veins. From the masson trichrome staining, fibrotic changes (Figure 3) were most pronounced in the TAA group.

Each sample of models of CCl_4 and TAA showed enlarged portal tracts and severe fibrosis deposition. Compared with model CCl_4, liver cirrhosis IV and fibrosis III were apparent respectively in 13 and 11 of 24 samples in model TAA. Fibrosis III and liver cirrhosis IV were apparent respectively in 6 and 11 of 18 samples in model CCl_4. The fibrosis scores of liver sections for both CCl4 and TAA models are shown in Table 3.

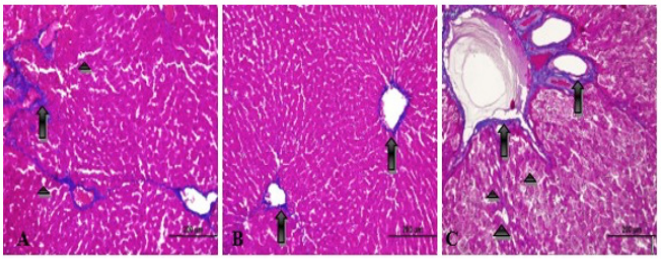

Figure 3: Liver histopathology of TAA-treated rats: Group 6.1. fibrosis (triangle shape presented vacuolization and acidophilus bodies in cytoplasm) tissue can be seen, it extended to the edge of liver lobule fibrosis (black thick arrows presented the fibrosis) A; group 7.1. hemorrhagic necrosis and inflammation B; group 8.2. triangle shape presented vacuolization and acidophilus bodies in cytoplasm and black thick arrows presented the fibrosis C; (×20).

Table 3. Histopathological semiquantitative scores of collagen deposition in the liver

Groups	n	0	I	II	III	IV
Control Groups	18	18	0	0	0	0
CCl_4 Model	18	0	0	1(5.6)	6(33.3)	11(61.1)
TAA Model	24	0	0	0	11(45.8)	13(54.2)

Values are expressed as n (%) ($p<0.001$).

The results showed that the degree of fibrosis scores were markedly increased on fourth, sixth and eighth weeks after cessation of CCl_4 and TAA. Fibrosis III and IV degrees, compared with the control group, were significantly increased and there were also marked differences between the two models ($p<0.001$).

DISCUSSION

Various animal models have been developed for liver diseases; fibrosis, CCl_4 and TAA are chemotoxin models [10,11]. Because of their applicability and reproducibility, they are the most commonly used chemotoxin models for inducing liver fibrosis [12]. Differences in the etiology and fibrosis degrees between these models have been reported in previous studies [13,14]. However, there are very few studies comparing the comprehensive properties of the two fibrosis models simultaneously [7]. Due to the dissimilar characteristics of chemotoxins, they were injected

intraperitoneally at different doses for different periods. In both models, fibrous septa transitions were seen around portal triads, and scoring showed severe fibrosis and liver cirrhosis in III and IV, respectively. These two hepatotoxic agents are known to be agents that increase oxidative stress causing damage to hepatitis [15]. ALT and AST values were elevated that inducing with CCl_4 forming the most potent free radical. Liver enzymes are markers of inflammation in hepatic damage [16]. These enzymes including ALT and AST have been elevated with chemotoxins. Both the serum ALT and AST were markedly increased at the end of fourth week after cessation of CCl_4 0,5 ml and also six rats have been seen severe fibrosis histopathologically at CCl_4 groups. At the end of the 6th and 8th weeks and group 5.1, it was observed that 11 rat had cirrhosis at these groups. ALT and AST enzyme levels have been elevated in TAA model. Although not as high as in CCl_4 groups, the number of rats that turn into cirrhosis is higher in this model. Histopathologically severe fibrosis was most common seen at group TAA 300 mg. At the both of models was seen liver enzymes increasing at the end of sixth week. It was observed pathologically differences for each model. This is because each chemotoxin has different properties. In addition, it has been shown that caused by the differences between the histological properties of liver effected, oxidative stress, liver enzymes and fibrotic changes. Among these models, it was observed that 300 mg TAA group had cirrhosis and 100 mg TAA group had severe fibrosis in the short term. In the CCl_4 model, it was seen histopathological changes which fibrosis characteristics on the 6th week at 0.5 ml CCl_4 group. When the other groups were examined, it was observed that the deaths due to the high doses and frequency of administration. In this study, 0.5 ml CCl_4 and 100 mg of TAA groups were found to be suitable to create an experimental animal model.

Limitation of Study: This study was pilot study. The small number of rats is an important limitation, in particular for the evaluation of the analysis of both groups. For more reliable results of each group, they should be examined in large-scale studies. We believe that this study should be a guide for larger ones to be carried out in the future.

CONCLUSION

In this study, it is provided to test the compounds that can be used as therapy for fibrosis or to interpret the background to evaluate fibrosis content. It will be a useful reference by saving time for researchers in the process of creating fibrosis model using animal models.

Conflict of Interest: The author has no conflict of interest related to this article.

Funding sources: This study was supported by Erciyes University, Scientific Research Project Unit (Project code: TSA-2016-6571).

Acknowledgement: We would like to thank Erciyes University, Genome and Stem Cell Center for allowing us to perform this study.

Ethics Committee Approval: All animal procedures and experimental protocols were approved by the Experimental Animals Ethics Committee of Erciyes University, Turkey (12/89-12/08/2012).

Peer-review: Externally and internally peer-reviewed.

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Arzu Hanım YAY 0000-0002-0541-8372	**Data collection and/or Processing, Final approval.**
Gülden BAŞKOL **0000-0001-7639-3099**	**Consept and/or Design, Analysis, Interpretation, Final approval.**
Tolga ERTEKİN 0000-0003-1756-4366	**Supervision and/or Critical Review, Final approval.**
Harun ÜLGER 0000-0003-3893-6341	**Consept and/or Design, Final approval.**