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1 **Consequences of Analgesics Use in Early Pregnancy: Results of tests on mice**

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30

31 **Abstract**

32 Self-medication during pregnancy continues to be an issue in developing countries due to
33 poor medical education. The most commonly used drugs are analgesics, mainly
34 acetaminophen (paracetamol, APAP) and, to a lesser extent, ketoprofen (KPF). The aim of the
35 study was to establish whether there are consequences of accidental use of these two drugs
36 during early embryogenesis. The experimental study was performed on 30 pregnant white
37 mice, divided into three groups: a first group to which APAP was administered, a second
38 group to which KPF was administered, and group 3 as a control group. At delivery, the baby
39 mice were examined, and after their first parturition, they were taken into study and examined
40 according to the established protocol. Macroscopic and microscopic examinations of the liver
41 and kidney were performed; liver and renal changes were recorded. Regarding the fertility,
42 the number of fetuses born to mothers that were administered APAP and KPF corresponded
43 to the normal values recorded in this species. Microscopic changes that were found in the
44 kidney were as follows: APAP group – necrosis of the urinary tube, vascular congestions and
45 vascular disorders; KPF group – ectasia, especially in the medullary cavity. Microscopic
46 hepatic changes showed in the APAP group – vascular congestions, vascular disorders and
47 hemosiderin deposits in the Kupffer cells; in the KPF group were found – discrete vascular
48 disorders consisting in sinusoidal capillary ectasia and vascular congestions, as well as the
49 presence of lymphocyte conglomerates. The aforementioned lesions indicate hepatic and renal
50 distress with variable degrees of severity, but they appear to be reversible (the longer the time
51 from the maximum effect of the drug the lower its toxicity).

52

53 **Key words:** paracetamol; ketoprofen; mice embryogenesis; microscopic changes; kidney;
54 liver.

55

56

57 **1. Introduction**

58 Despite the development and diversification of contraception methods and particularly of
59 prenatal diagnosis of congenital malformations, each year there are still a large number of
60 preterm and full-term newborns who are born with these conditions, due to the high
61 proportion of cases of social difficulties and the absence of pregnancy checkups.

62 Meanwhile, the tendency toward self-medication with analgesics is a problem worldwide.
63 Paracetamol (APAP), under various dosage forms and trade names, is one of the products
64 most used, even during the pregnancy period. To a lesser extent ketoprofen (KPF) is also
65 used by pregnant women (Andrade et al., 2004; Bayene and Beza, 2018). APAP use is so
66 widespread among the population that studies are showing its ubiquitous presence in human
67 urine (Modick et al., 2014).

68 The occasional self-medication by pregnant women, when they are not aware of their
69 pregnancy, is an additional aspect of the above situation. Consequences may be more severe if
70 self-medication occurs during embryogenesis, in cases when pregnancy has not yet been
71 identified. These cases may be referred to as accidental use of such substances by the
72 pregnant women.

73 Although many studies confirm the effects of long-term exposure of embryos to these drugs
74 (Adjei, et al., 2008; Feldkamp et al., 2010; Kristensen et al., 2011; Rebordosa et al., 2008),
75 as do many experimental studies on mice and rats (Burdan et al., 2001; Kristensen et al.,
76 2011), data establishing the correlations between short-term occasional use of these
77 substances and the emergence of abnormalities are still insufficient.

78 Most problems occur with so-called teratogenic drugs that cross the placenta and enter the
79 placental fetal circulation. Sometimes the effects of drugs on the fetus are known only after
80 many years. Thalidomide, for example, used in Europe in the 1960s, resulted in numerous
81 serious birth defects, such as missing or incompletely formed limbs (Gilbert-Barnes, 2010;

82 Vargesson, 2015). It had been put on the market at the end of the 1950s and was used for a
83 considerable period of time, until it was banned and withdrawn from the market in the UK, at
84 the end of 1961, and in 1962 almost worldwide (Gilbert-Barnes, 2010).

85 For medicines / drugs, the list of agents shown to be potentially teratogenic is surprisingly
86 small (Mitchell et al., 2011). The term “teratogen” is attributed to any environmental factor
87 that causes permanent abnormalities in the structure, function or development of the embryo
88 or fetus (Gilbert-Barnes, 2010). In the first two weeks of pregnancy (the pre-embryonic
89 stage), the embryo appears to be resistant to the action of teratogenic factors, but a significant
90 exposure can be lethal. Fetal malformations are caused by certain medications, narcotics,
91 chemicals (Abdel-Daim et al., 2018 a and b), maternal conditions or diseases (Stoicescu et al.,
92 2017; Tataru et al., 2019), including infections, that can affect the normal intrauterine
93 development of the fetus. A connection between the dose of medication intake and its effect
94 has been found for animals or humans, a greater intake during pregnancy determining more
95 severe phenotypic effects on the fetus (Frias and Gilbert Barnes, 2008).

96 During the first three to eight weeks, there is a period of maximum susceptibility to the action
97 of teratogenic agents because all the major organs are formed now (organogenesis). Most fetal
98 malformations occur in this stage of pregnancy (although less than 1% of total congenital
99 malformations are caused by human teratogenic drugs) (Mazzu-Nascimento et al., 2017).

100 The induction of fetal malformations by these agents depends on the maternal and paternal
101 genetic predisposition, the stage of fetal development at the time of exposure, the route of
102 administration and the duration of exposure to the teratogenic agent (Brent, 2001; Gilbert-
103 Barnes, 2010). It is crucial to develop medical education programs targeting all social
104 categories, which inform on the possibilities of family planning and the importance of proper
105 dispensation of all medications during pregnancy and of avoiding self-medication (Bayene
106 and Beza, 2018). Such programs would help reduce the number of unwanted pregnancies,
107 and—if medications are dispensed properly during pregnancy—exposure to a number of

108 teratogenic factors would be avoided, thereby leading to a significant decrease in the
109 percentage of serious birth defects/malformations.

110 The purpose of this experimental study was to identify the effects of the maximum tolerated
111 dose of certain drugs during embryogenesis. It was intended to establish a correlation between
112 acetaminophen or ketoprofen and disorders in the first part of pregnancy, during
113 embryogenesis. Our research, which was performed on female mice during pregnancy,
114 examined the possibility of congenital disorders.

115

116 **2. Materials and methods**

117 **2.1. Study design**

118 Experiments were performed on white mice for the following reasons: they have a high
119 reproduction potential, thereby ensuring the number of animals needed; they are easy to
120 handle; they can easily be put into homogenous groups (by weight, age); they do not require
121 large spaces or special care conditions; and they are resistant to infections and stress from
122 experiments. Thirty pregnant white mice were selected and divided into three groups: group A
123 – to which APAP was administered, group B – to which KPF was administered, and group C
124 – the control group.

125 **2.2. Ethical Statement**

126 This research was performed in compliance with the European Convention principles for the
127 protection of vertebrate animals used in experimental and other scientific purposes, adopted in
128 1986, in Strasbourg (ECPVAEOSP, 1986), to the 2010/63/EU Directive of the European
129 Parliament and of the European Council on the protection of animals used for scientific
130 purposes, adopted on September 22, 2010, and in accordance with laboratory good practices.

131 The mice were kept under optimal microclimate conditions, locked in cages with feeders and
132 waterers, in the same room. The study was approved by the Ethical Local Commission of the

133 Pius Brânzeu Center for Laparoscopic Surgery and Microsurgery of the Victor Babeș
134 University of Medicine and Pharmacy Timisoara (reference number 680/12.04.2006).

135 **2.3. Technique of medical substances administration**

136 After weighing, all animals were caught by the tail, pulled out of the scale box and placed on
137 a smooth drape. The animals were then covered with another drape, held firmly but delicately,
138 and marked on the tail with a permanent marker: blue for APAP and red for KPF. The
139 posterior girdle was lifted to expose the hypogastric region, where the drug dose (calculated
140 based on the weight obtained by scaling and presented in **Table 1**), was injected
141 intraperitoneally by means of a fine-needle syringe; the procedure was repeated strictly on
142 each animal, for three consecutive days. The substances were administrated to the mice
143 between the fifth and seventh gestation days. This time interval was chosen because nidation
144 during embryogenesis in mice occurs 4.5 days after conception, corresponding to the early
145 second week of human embryogenesis, when nidation occurs. Day 7 of embryogenesis in
146 mice matches the middle of the third week in human embryogenesis (Day 17), when the
147 delayed menstrual cycle may lead the woman to perform a pregnancy test and to stop self-
148 medication in the event of positive diagnosis (Hill, 2019). The three days of the mouse
149 embryogenesis during which drugs were administered (5th to 7th days) are equivalent to 10
150 days of human embryogenesis (7th to 17th days) (Hill, 2018; Theiler Staging Criteria, 2019).

151 The mice were observed until the end of gestation and kept under optimal microclimate
152 conditions. After delivery, the baby mice were examined, to assess the possible consequences
153 of drug administration on fertility. After the first reproductive cycle, the mice were euthanized
154 and macroscopic and microscopic examinations of the liver and kidney performed.

155 Macroscopic changes of liver and kidneys were assessed by comparing them to the aspect of
156 organs harvested from the control group, focusing on their appearance upon inspection,
157 preservation of shape and surface uniformity, sizes, and consistency at palpation. After the

158 macroscopic examination of the harvested organs, microscopic examination using the
159 hematoxylin-eosin (H.E.) staining method was performed.

160 **2.4. Statistical analysis**

161 To gather the database, Microsoft Excel (version 2013) was used, and for the statistical
162 analysis two different programs were available: SPSS (version 17) for the statistical
163 significance and Microsoft Excel for the figures and the descriptive statistics. A normality test
164 was applied, in our case a Shapiro–Wilk test (because our study group consisted of less than
165 50 subjects) to see if our data were normally distributed. In order to describe the database, the
166 central tendency and the dispersion parameters were calculated. For the statistical
167 significance, an ANOVA test was used.

168

169 **3. Results**

170 With regard to the *fertility* of the females exposed to intrauterine action of the drugs, no
171 changes were recorded in comparison with the control group. The number of fetuses born to
172 mothers who were administered APAP and KPF corresponded to the normal values recorded
173 in this species, 6-10 per litter apparently being normal. Their growth and development were
174 monitored up to the sexual maturity and the birth of the first generation of babies (normal
175 growth as regards the number and morphology of animals).

176 Upon *morphological examination* of the subjects, no differences were recorded in comparison
177 with the control group. Macroscopic examination of liver and kidneys indicated no
178 abnormalities in the animals exposed to intrauterine action of the drug in comparison with the
179 control group.

180 **Microscopic examination of the organs**

181 *APAP group*

182 Microscopic changes in the kidney (after administration of the drug) were found in the form
183 of necrosis of the urinary tube, desquamation of epithelium, vascular congestion and vascular

184 disorders. At the same time, microscopic hepatic changes showed vascular congestion,
185 vascular disorders and hemosiderin deposits in the Kupffer cells.

186 *KPF group*

187 The microscopic renal changes after administration of KPF were vascular disorders consisting
188 of ectasia, especially in the medullary cavity. In the liver, discrete vascular disorders were
189 observed, consisting in sinusoidal capillary ectasia and vascular congestion, as was the
190 presence of lymphocyte conglomerates. Such lesions indicate hepatic and renal distress in the
191 animals undergoing intrauterine exposure to APAP and KPF during embryogenesis. Lesions
192 were severe to variable degrees but appear to be reversible. The histopathological changes
193 described were also observed in overdose situations in animals such as dogs and cats which
194 for instance developed transient renal and / or hepatic impairment. In addition, the changes
195 assessed through biopsy puncture were less pronounced since the period between
196 administration and puncture was higher. Basically, if biopsy puncture was made at peak effect
197 of the active substance, the changes would more pronounced: the longer the time from the
198 maximum effect of the drug the lower its toxicity. The most representative aspects of the
199 lesions are presented in **Figures 1 and 2**.

200 The main observation that can be extracted from the descriptive statistical analysis is that the
201 mean weight of our subjects is almost the same regardless of whether they received either
202 drug (APAP or KPF) or no drug. The mean values for each mouse over the three tested days,
203 for weight and for the drug doses, were obtained (**Table 2**).

204 Later, the Shapiro–Wilk normality test was applied. The $p > 0.05$ value assures the use of the
205 ANOVA test for establishing the statistical significance between the tested groups. The main
206 part of the analysis consisted in applying the ANOVA Two Factor Without Replication test in
207 order to see if there were any differences among the weight or the drug doses over the three
208 tested days, or if there were any differences among the ten tested mice for either drug.

209 Therefore, four different ANOVA Two Factor Without Replication tests were performed, and
210 their results are presented in **Tables 3** (for APAP) and **4** (for KPF).

211 In the first test, which compared the mice weight over the three days, considerably significant
212 differences ($p < 0.001$) were obtained in the following two scenarios: when the differences
213 among the ten mice were examined and when the differences between the studied days were
214 examined. In the second test, concerning the APAP dose, extremely significant differences
215 were obtained for both scenarios: when the differences among the subjects were tested and
216 when the differences among the studied days were tested. The same observation holds true for
217 the KPF mice group, under the same circumstances, regarding the $p < 0.001$ value (**Tables 3**
218 and **4**).

219 A final hypothesis was tested at the end of this study. We wanted to see if any differences
220 could be found in the weights of the mice used in this experimental study, in all three groups
221 (mice with APAP, with KPF and drug-free). Therefore, an average value for weight over the
222 three days studied was calculated. Application of the ANOVA Single Factor test showed that
223 there were no statistically significant differences ($p = 0.883$, which is > 0.05) among the
224 weights of the mice in all tested groups. In short, the results showed that the drugs did not
225 influence weight dynamics (**Figure 3**).

226

227 **4. Discussion**

228 Some studies have reported side effects on fetuses from APAP use during pregnancy, as seen
229 by the presence of congenital malformations clinically diagnosed at birth (Feldkamp et al.,
230 2010). Thielea et al. (2013) published one of the most complex analyses on the short- and
231 long-term effects of APAP use during pregnancy. Data in the field also includes information
232 about the possible onset of gastroschisis. A retrospective study which included 206 cases of
233 gastroschisis reported an increased risk of this malformation in the event of APAP use during
234 pregnancy (odds ratio = 1.5, 95% confidence interval: 1.1, 2.2) (Werler et al., 2002). Other

235 authors have reported an increased odds ratio for neural tube defects (Lynberg et al., 1004)
236 and an increased odds ratio for congenital cryptorchidism, especially in the second trimester,
237 and reduced testosterone production in *ex vivo* fetal rat testes (Kristensen et al., 2011; Jensen
238 et al., 2010). In a study published in 2014 and conducted on 64,322 live-born children and
239 mothers enrolled in the Danish National Birth Cohort during 1996-2002, Liew et al. (2014)
240 show that prenatal exposure to APAP action increases the risk of behavioral disorders or
241 hyperkinetic syndrome.

242 Studies regarding the effects of KPF use during pregnancy are fewer in number. A study on
243 drug prescriptions during pregnancy reports on KPF administration during the last trimester of
244 pregnancy (Garcia et al., 2000). Other data concluded on the consequence of KPF use during
245 pregnancy on a group of 11 newborns from mothers who experienced adverse effects to this
246 drug. Ten of them had renal dysfunction and three deaths were recorded (Llanas et al., 1996).
247 A case of a newborn with kidney failure following KPF administration to the mother during
248 the last four days of pregnancy was also presented (1991).

249 In this study, administration of APAP or KPF for a short period of time, between the 5th to 7th
250 days of gestation period in mice, does not influence pregnancy evolution. Mice resulting from
251 such pregnancies have normal morphological and behavioral development in comparison with
252 the control group, and the fertility of these animals has the values characteristic to the species.
253 Macroscopic examination of liver and kidneys of animals exposed to the action of these
254 substances during embryogenesis did not show pathological aspects.

255 Our research did not find any congenital malformations, behavioral disorders or hyperkinetic
256 syndrome, cryptorchidism or affected fertility in mice which, in the early pregnancy period,
257 were exposed, for a short time, to one of these drugs (APAP or KPF). However, this paper
258 mentions some hepatic and renal microscopic changes noted in standard histologic
259 examinations, but with no vital risk. But these changes call for more in-depth studies
260 regarding the accidental use of APAP and/or KPF during the first period of pregnancy. Thus,

261 there is a clear need for complex educational programs dedicated to reducing self-medication
262 among the general population, especially among fertile or pregnant women (in order to
263 drastically reduce the phenomenon of self-medication in the case of undiagnosed pregnancies)
264 (Bungau et al., 2015). Reducing self-medication is essential along with taking into account the
265 threats caused to the environment by increased drug consumption, especially those commonly
266 in use such as APAP and KPF. For example, the disposal of expired or unused drugs into
267 waste or landfilled waste exacerbates their concentration in the environment (Cycońet al.,
268 2016). Since paracetamol is among the most frequently used nonprescription drugs to reduce
269 fever and relieve pain it is often found in surface water, sewage treatment plants (Taschina et
270 al., 2017) and drinking water.

271 APAP concentrations in aquatic environments increased over the last years, since they are not
272 completely removed in wastewater treatment plants, and their residues may be released
273 through effluents into the aquatic environment (Al-Kaf et al., 2017). The continuous dumping
274 of non-steroidal anti-inflammatory drugs is of concern since it is practically impossible to
275 assess their concentration in the environment along with the potential negative impacts on
276 living organisms in the future (Cycońet al., 2016).

277

278 **5. Conclusions**

279 Our study revealed that administration of APAP or KPF drugs does not influence the weight
280 dynamics of mice during pregnancy; they developed normally, and no differences are
281 observed between mice administered APAP or KPF and other healthy mice. Changes /
282 abnormalities that occur can be seen only at the level of the kidney and liver and are visible
283 just in a histological examination. At present, we cannot determine whether these
284 abnormalities (caused by developing tissue damage) are due to the direct *in utero* toxicity of
285 the analgesic, its metabolites or other factors. We believe that further studies are needed to
286 determine the mechanisms leading to these lesions.

287

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293

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400 **Figure legends**

401
402 **Figure 1.** (a) Urinary tube necrosis with epithelium desquamation, section in HE staining of
403 kidney, detailed image, 40x; (b) Erythrocyte agglutinations indicating vascular congestion,
404 section in HE staining of kidney, detailed image, 40x; (c) Vascular disorders consisting of
405 vascular congestions, section in HE staining of liver, detailed image, 40x; (d) Congestions,
406 hemosiderin deposits in the Kupffer cells, section in HE staining of liver, detailed image, 40x.
407

408 **Figure 2.** (a) Vascular disorders consisting in ectasia, section in HE staining of kidney,
409 detailed image, 40x; (b) Vascular disorders consisting in regional ectasia of the sinusoidal
410 capillaries and passive vascular congestions, lymphocyte conglomerate, section in HE
411 staining liver, detailed image, 40x.

412

413 **Figure 3.** The weight distribution within the 3 groups

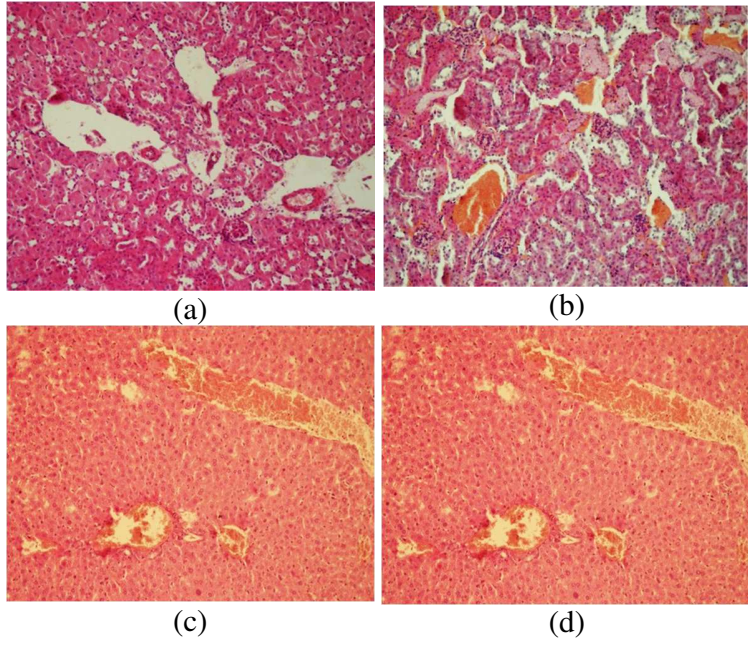
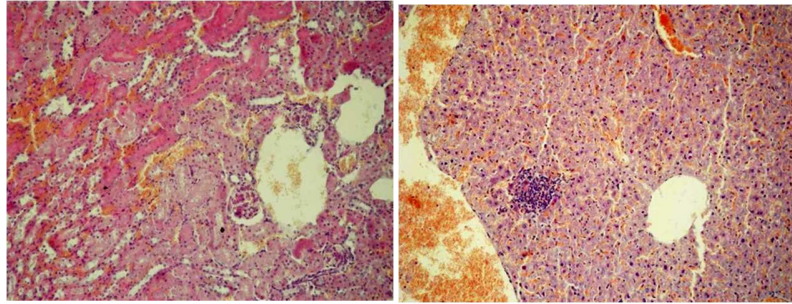


Figure 1 (a); (b); (c); (d).



(a)

(b)

Figure 2 (a); (b).

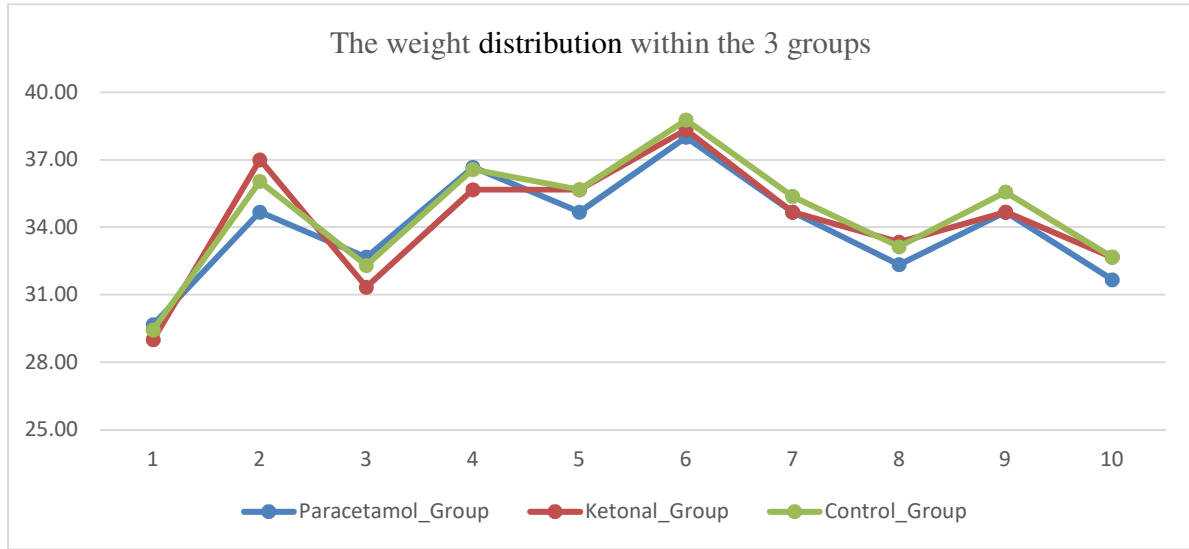


Figure 3.

Table 1. APAP and KPF doses/animal weight/day (50 mg/kg/day and 5 mg/kg/day respectively)

Animal No.	Day	Weight (g)	Dose (mg)	Weight (g)	Dose (mg)
		APAP		KPF	
1	I	28	1.4	27	0.135
	II	29	1.45	29	0.145
	III	32	1.6	31	0.155
2	I	34	1.7	36	0.18
	II	33	1.65	37	0.185
	III	37	1.85	38	0.19
3	I	29	1.45	29	0.145
	II	33	1.65	31	0.155
	III	36	1.8	34	0.17
4	I	35	1.75	33	0.165
	II	36	1.8	36	0.18
	III	39	1.95	38	0.19
5	I	34	1.7	34	0.17
	II	34	1.7	35	0.175
	III	36	1.8	38	0.19
6	I	37	1.85	36	0.18
	II	38	1.9	38	0.19
	III	39	1.95	41	0.205
7	I	32	1.6	32	0.16
	II	35	1.75	35	0.175
	III	37	1.85	37	0.185
8	I	29	1.45	29	0.145
	II	33	1.65	33	0.165
	III	35	1.75	35	0.175
9	I	32	1.6	32	0.16
	II	35	1.75	35	0.175
	III	37	1.85	37	0.185
10	I	29	1.45	29	0.145
	II	32	1.6	32	0.16
	III	34	1.7	34	0.17

Table 2. The main tendency and dispersion parameters for the mean values (the average values computed for the 3 tested days) for the mice weight and APAP/KPF doses

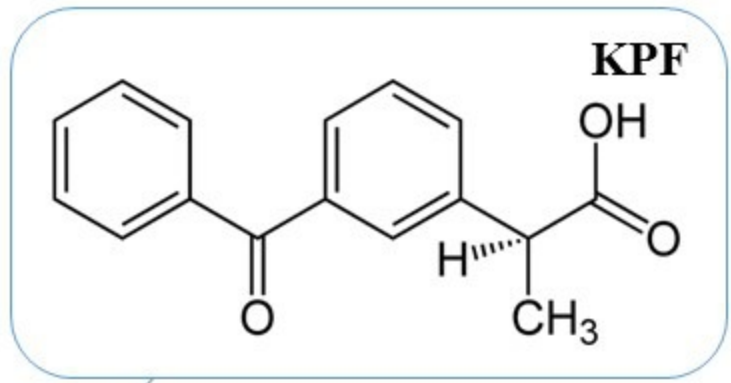
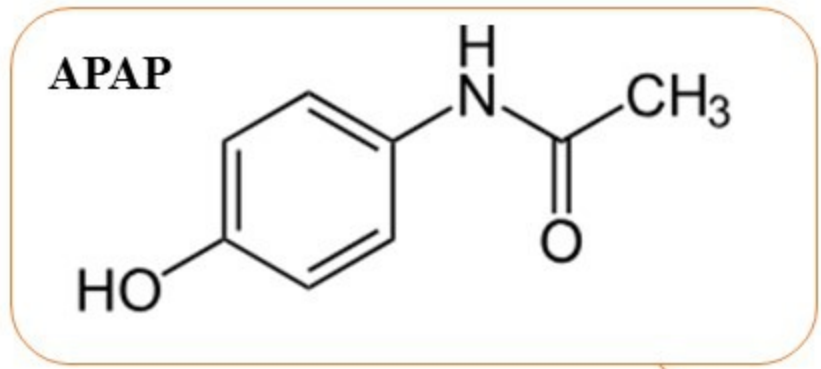
Statistics	Mean Weight Group 1	Mean APAP dose	Mean Weight Group 2	Mean KPF dose
Mean	33.97	1.70	34.23	17.83
Standard Error	0.77	0.04	0.87	0.41
Median	34.67	1.73	34.67	18.20
Mode	34.67	1.73	35.67	18.20
Standard Deviation	2.44	0.12	2.75	1.28
Sample Variance	5.96	0.01	7.56	1.64
Kurtosis	-0.05	-0.05	0.16	-0.05
Skewness	-0.11	-0.11	-0.51	-0.11
Range	8.33	0.42	9.33	4.38
Minimum	29.67	1.48	29.00	15.58
Maximum	38	1.90	38.33	19.95
Sum	339.67	16.98	342.33	178.33
Count	10	10	10	10

Table 3. The ANOVA Two-Factor Without Replication test applied on the APAP injected mice group for weight and for drug dose.

Source of variation	SS	df	MS	F	F crit.
Weight					
The analysis on mice	160.9667	9	17.88519	16.82578	2.456281149
The analysis on the 3 studied days	92.86667	2	46.43333	43.68293	3.554557146
Error	19.13333	18	1.062963		
Total	272.9667	29			
Drug dose					
The analysis on mice	0.402417	9	0.044713	16.82578	2.456281
The analysis on the 3 studied days	0.232167	2	0.116083	43.68293	3.554557
Error	0.047833	18	0.002657		
Total	0.682417	29			

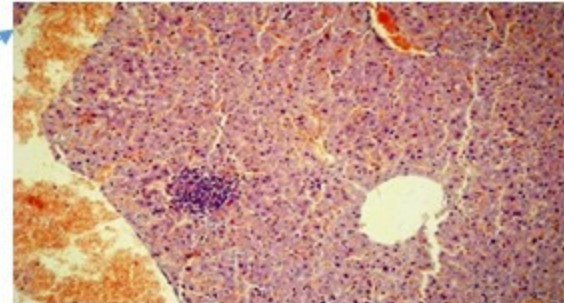
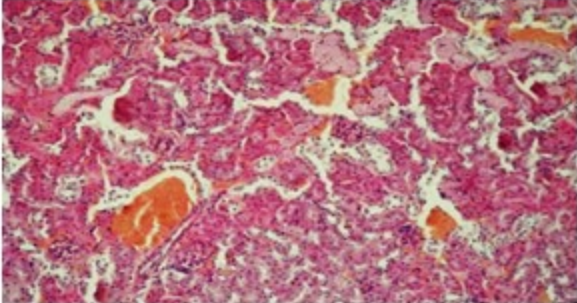
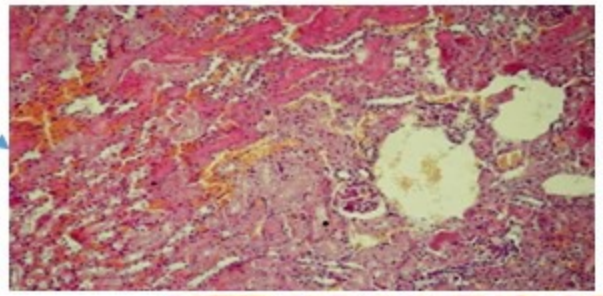
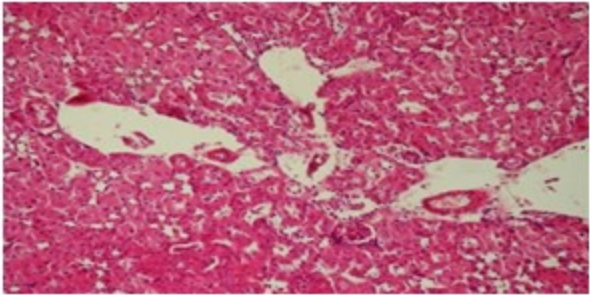
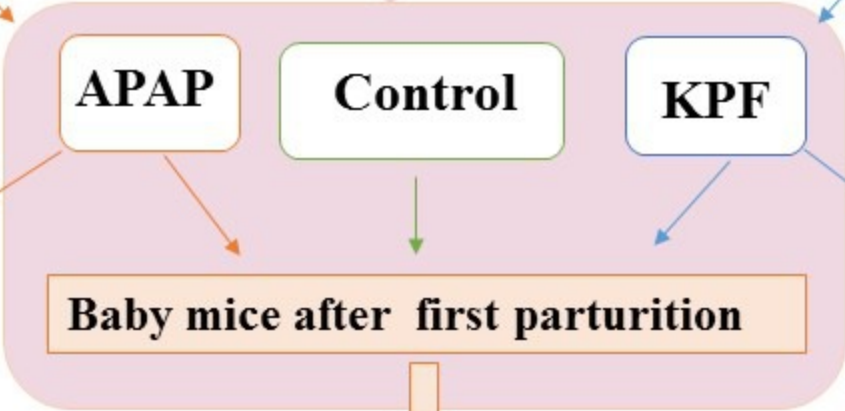
Table 4. The ANOVA Two-Factor Without Replication test applied on the KPF injected mice group for weight and for drug dose.

Source of variation	SS	df	MS	F	F crit.
Weight					
The analysis on mice	223.6333	9	24.84815	59.90179	2.456281
The analysis on the 3 studied days	105.8667	2	52.93333	127.6071	3.554557
Error	7.466667	18	0.414815		
Total	336.9667	29			
Drug dose					
The analysis on mice	0.005591	9	0.000621	59.90179	2.456281
The analysis on the 3 studied days	0.002647	2	0.001323	127.6071	3.554557
Error	0.000187	18	1.04E-05		
Total	0.008424	29			



30 pregnant white female mice

3 groups



Fertility
Morphological examination
Microscopic examination of the organs

No congenital malformations, behavioral disorders or impaired fertility in mice (to APAP or KPF influence) were found. Some liver and kidney microscopic changes have been described in the standard histological examination but without vital risk.