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Research Article

Genotoxic and Cytotoxic Effects of the Aglepristone, A Progesteron Antagonist, in Mid-gestation Pregnancy Termination in Rabbits

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Abstract

Aglepristone is an antiprogestin using for pregnancy termination in veterinary medicine. The information about side effects of aglepristone is limited. The aim of the study was to investigate cytotoxicity and genotoxicity of aglepristone in mid-gestation pregnancy termination in rabbits. Fifteen New Zealand White rabbits were used and pregnant does were randomly divided into three groups. Group I (n=5) was treated with saline as the control. The does in group II (n=5) and group III (n=5) were treated with aglepristone (10 mg/kg) on 15th day and 15th-16th days of pregnancy, respectively. The rabbits were sacrificed by guillotine 24 h after last treatment. Bone marrow and blood samples were immediately collected. Cytotoxic and genotoxic potential were tested by micronucleus and Comet assays. No genotoxicity and cytotoxicity were found in micronucleus test with single aglepristone administration. In contrast, two consecutive treatments of aglepristone showed high genotoxic and cytotoxic effects on bone marrow in animals. While comet assay of blood samples did not show any significant difference between groups; the results from comet assay of bone marrow cells showed the single injection of aglepristone did not induce any DNA damage but two injections group increased the DNA damage.

Keywords: *Aglepristone, Cytotoxic, Genotoxic, Micronucleus test, Comet assay*

Tavşanlarda Orta Dönem Gebelikleri Sonlandırılmasında Kullanılan Bir Progesteron Antagonisti Olan Aglepriston'un Genotoksik ve Sitotoksik Etkileri

Özet

Aglepriston, veteriner hekimlikte gebeliklerin sonlandırılmasında kullanılan bir antiprogestindir. Aglepriston'un yan etkileri ile ilgili bilgiler sınırlıdır. Çalışmamızın amacı; orta gebelikte gebeliği sonlandırılan tavşanlarda aglepriston'un potansiyel sitotoksik ve genotoksik etkilerinin araştırılmasıdır. Çalışmada 15 Yeni Zelanda beyaz tavşanı kullanılmıştır ve gebe tavşanlar rastgele üç gruba ayrıldı. I. Grup (n=5) kontrol grubu olarak tuz çözeltisi ile muamele edildi. II. Grup (n=5) gebeliğin 15. gününde aglepriston (10 mg/kg) enjekte edilen III. Grup (n=5) ise gebeliğin 15. ve 16. günlerinde aglepristone enjekte edilen gruptu. Tavşanlar son enjeksiyonlardan 24 saat sonra giyotin ile sakrifiye edilerek hızlı bir şekilde kemik iliği ve kan örnekleri alındı. Sitotoksik ve genotoksik potansiyel mikroçekirdek ve komet yöntemleri ile araştırıldı. Tek aglepriston uygulaması ile gebeliği sonlandırılan tavşanlarda mikroçekirdek testi ile herhangi bir sitotoksik ve genotoksik etki belirlenemedi. Bunun tersine iki aglepriston uygulanan tavşanların kemik iliği hücrelerinde yüksek sitotoksik ve genotoksik etki belirlendi. Komet yönteminde kullanılan kan örneklerinde gruplar arasında bir fark belirlenemedi. Kemik iliği hücrelerinin kullanıldığı komet yönteminde tek enjeksiyon grubunda herhangi bir DNA hasarı belirlenmemesine rağmen çift enjeksiyon grubunda DNA hasarının arttığı belirlendi.

Anahtar sözcükler: *Aglepriston, Sitotoksik, Genotoksik, Mikroçekirdek testi, Komet yöntemi*

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INTRODUCTION

Aglepristone, developed as antiprogestin, has high potential activity in blocking action of progesterone in veterinary therapy. Anti-progesterone activity of the synthetic steroid has been clinically tested in small animal veterinary practices. Based on the clinical experiences, aglepristone can be safely used in specific situations such as termination of unwanted pregnancies, induction of parturition, treatment of pyometra and fibroadenomatosis in dogs and cats [1-6]. The successful clinical features in induction abortion during mid-gestation and implantation have been thoroughly investigated in rabbits [7-9]. Progesterone antagonists have similar structure and show high binding affinity to the receptor with hydrophobic side chain at C17. The administration of aglepristone leads conformational changes leading to a suppression of transcription at additional aromatic ring at C11 with a dimethyl-amino group, resulting incomplete signaling cascade and non-biologic effect of progesterone [1,10,11].

In general, *in vivo* genotoxicity test systems contain micronucleus (MN) and comet tests in experimental rodent models [12]. Micronuclei arise from chromosome breakage (a result of clastogenic activity) or lagging chromosomes (due to aneugenic activity) in the anaphase stage of cell division. Generally, mammals may produce spontaneously micronucleated cells at background level and their presence might increase when organisms are exposed to genotoxic compounds [13,14]. MN test can be conducted from peripheral blood samples, liver and bone marrow [15-18]. Especially, MN tests are carried out on bone marrow samples in adult rabbits, because the spleen quickly removes damaged micronucleated erythrocytes from the peripheral blood [19]. DNA damage can be detected by embedding single cells in agarose gel and staining with a fluorescent-DNA binding dye after electrophoresis, named single-cell gel electrophoresis (comet assay) method [20].

Mifepristone, lilopristone and onapristone are the counterparts of aglepristone. There is no information about the genotoxicity of mifepristone but lilopristone and onapristone were studied with negative results [21]. On the other hand, many exogenous hormonal steroids may cause genotoxic or cytotoxic effects and chromosome breakage [21]. Some natural estrogens, estradiol and estrone and medroxyprogesterone group of synthetic progestins with cyproterone acetate are prominent examples that are gene mutation or genotoxic activity in different tests [22].

To our knowledge, there is no information available about the toxicity of aglepristone. So the objective of this study was to investigate possible genotoxic or cytotoxic effects of aglepristone by MN test and comet assay in mid-gestation termination in does.

MATERIAL and METHODS

Study Design and Animals

Fifteen healthy, 12 months old, New Zealand White rabbits weighing 2.800-3.400 g were housed individually under day-light conditions. All does were fed a standard commercial dry food once (5 g/100 g BW) daily and given water *ad libitum*. Approval of the Ethical Committee of the Uludag University for using the animals was obtained (2013-01/06). The does were brought to a cage of a fertile buck and the first mating was observed. Next day after mating was recorded as first day of pregnancy. Pregnancy confirmation was carried out by ultrasonographic examination (5-7.5 MHz linear array transducer; Siemens Sonoline Prima, Siemens Medical System, USA) on day 14 after mating. The does were examined in dorsal recumbency in order to detect gestational sacs and fetal heart beats. The pregnant does were randomly divided into three groups. Pregnant does in group I (n=5) were injected the same volume of 0.9% sodium chloride solution (Eczacibasi, Baxter, Turkey) whereas the animals in group II (n=5) and group III (n=5) were treated with aglepristone at a dose of 10 mg/kg (Alizin®,Virbac, Germany) once on day 15 post-mating and twice on days 15 and 16 post-mating, respectively.

Micronucleus Test

Micronucleus test was used for the detection of genetic damage that probably induced by compounds [23]. The rabbits were sacrificed by guillotine. Femur was immediately removed from animals with forceps and scissors and bones were cleaned off adhering tissues. The epiphysis of femur was dissected; bone marrow was taken from medullar canal and placed in 2 ml fetal bovine serum. Then bone marrow was thoroughly mixed to obtain a fine suspension and centrifuged at 1000 rpm for 5 min. The pellet was then re-suspended in fresh bovine albumin and smears were prepared on clean glass slides. The smear slides were kept dark for overnight, stained with May-Gruenwald and Giemsa at pH 6.8. For the determination of the frequency of micronucleated polychromatic erythrocytes (MNPCEs), 1000 polychromatic erythrocytes (PCEs) per animal were analyzed by light microscopy. Cytotoxicity was assessed by scoring the relative proportion of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). This ratio was determined by counting a total of 2000 erythrocytes for each animal.

The Single-cell Gel Electrophoresis (Comet) Assay

Rabbit bone marrow was taken from medullar canal from both femur and suspended in chilled PBS; blood sample was taken from ear vein and suspended in PBS. The cell suspensions were then mixed with an equal amount of 1% low melting agarose and the slides were prepared [24]. The slides were kept overnight at 4°C in lysis solution (2.5

M NaCl, 100 mM Na_2EDTA, 10 mMTris (pH 10), with 1% Triton X-100 (added just before use). Slides were subjected to DNA unwinding for 20 min and then electrophoresis was performed at 0.7 V/cm and 300 mA at 4°C for 25 min in freshly prepared electrophoresis buffer (1 mM EDTA sodium salt and 300 mM NaOH). After electrophoresis the slides were neutralized with Tris buffer and were stained with 20 µg/ml ethidium bromide. Slides were scored at a final magnification of 400x using an image analysis system (Microsystem®, Istanbul, Turkey) attached to a microscope (Nikon Eclipse 80i) equipped with a fluorescence attachment of a CCD camera. The comet parameters were used to measure DNA damage in the cells was Olive Tail Moment (OTM), tail DNA (%) and tail length (µm). Images from 100 random cells (50 from each replicate slides) were analyzed for each experiment.

Statistical Analysis

Data are expressed as mean ± SEM (standard error of the mean) in all cases. Five rabbits were tested in each group. Data were analyzed using one-way ANOVA followed by Tukey test. A P value of less than 0.05 was considered significant.

RESULTS

The means, standard errors, and significance of the MNPCE and PCE/NCE frequencies were given in *Table 1* and photographic presentation of micronucleated polychromatic erythrocyte was shown in *Fig. 1*. When single aglepristone injected rabbits (Group II) was compared with the control (Group I), no significant differences (P>0.05) were observed in the number of MNPCE and PCE/NCE ratio. Two consecutive injections of aglepristone (Group III) resulted significantly higher MNPCE number (P<0.0001) and lower PCE/NCE ratio (P<0.0001) compared with control animals. When animals in Group II were compared with the animals in Group III, significant differences were observed in the number of MNPCE (P<0.0001) and ratio of PCE/NCE (P<0.001).

The comet assay results were presented in *Table 2* and photographic presentation of slides were shown in *Fig. 2*. There was no significant difference in single aglepristone injected group (Group II) compared with control group (Group I) for OTM, tail % DNA and tail length results (P>0.05). Two injections group (Group III) were shown statistically significant elevation of OTM (P<0.0001), tail % DNA (P<0.0001) and tail length frequencies (P<0.0001).

DISCUSSION

In the present investigation, no genotoxicity and cytotoxicity were detected in MN test with single aglepristone

Table 1.*Genotoxic and cytotoxic effect of aglepristone in micronucleus test*
Tablo 1. *Aglepristone'nin mikroçekirdek testindeki genotoksik ve sitotoksik etkileri*

Groups	n	MNPCE	NCE/PCE
Group I	5	5.80±2.79	0.98±0.40
Group II	5	7.40±3.06	0.92±0.38
Group III	5	15.80±6.55 [a,b]	0.75±0.32 [a,c]

One pregnant rabbit per treatment; data are expressed as mean ± standard error; ***n:*** *number of rabbits;* ***MNPCE:*** *micronucleated polychromatic erythrocytes;* ***PCE:*** *polychromatic erythrocytes;* ***NCE:*** *normochromatic erythrocytes;* [a] *P<0.0001 vs. Group I;* [b] *P<0.0001 vs. Group II;* [c] *P<0.01 vs. Group II*

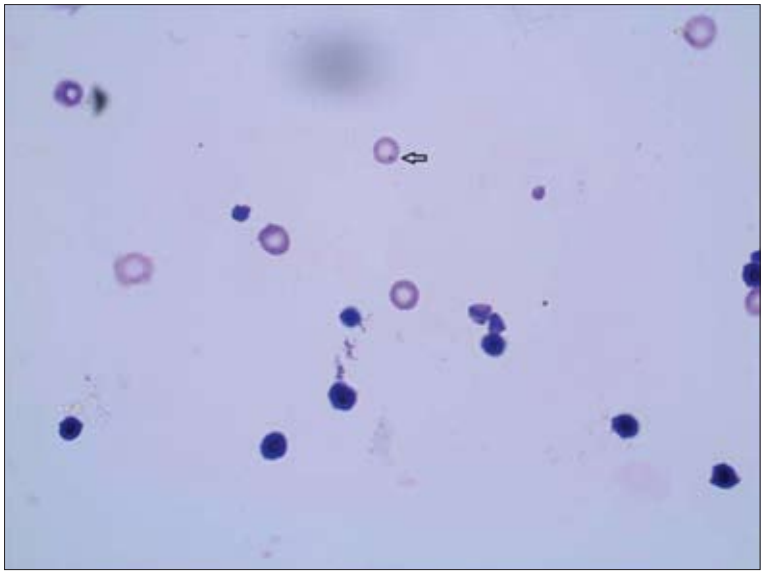

Fig 1. Photographic image of example of MN test under light microscopy (x100 magnification). *Arrow* indicated a MN in PCE in Group II (single injection group)

Şekil 1. Işık mikroskobualtında (X100) MN testi örneğinin fotoğrafik görseli. *Ok* Grup II (tek enjeksiyon grubu)'de PCE içerisindeki MN'yi işaret etmektedir

Table 2. *DNA damaging effect of aglepristone in comet assay*
Tablo 2. *Aglepristone'nin komet yöntemindeki DNA hasar etkileri*

Statistical Data	Group I			Group II			Group III		
	Tail Length (µm)	Tail % DNA	OTM	Tail Length (µm)	Tail % DNA	OTM	Tail Length (µm)	Tail % DNA	OTM
Mean^{BM}	7.81	6.90	0.39	7.82^{a}	7.39^{a}	0.45^{a}	26.20^{b}	17.4^{b}	3.84^{b}
SE	0.17	0.31	0.02	0.17	0.35	0.02	0.56	0.49	0.16
Mean^{BL}	10.07	5.13	0.175	10.2^{a}	5.38^{a}	0.21^{a}	10.43^{a}	5.39^{a}	0.21^{a}
SE	2.95	1.74	0.143	2.97	3.01	0.26	2.68	1.78	0.09

One pregnant rabbit per treatment; data are expressed as mean ± standard error; ***OTM:*** *Olive Tail Moment;* ***SE:*** *Standard Error;* ***BM:*** *Bone Marrow;* ***BL:*** *Blood;* ^{a} *P>0.05 vs. Group I;* ^{b} *P<0.0001 vs. Group I*

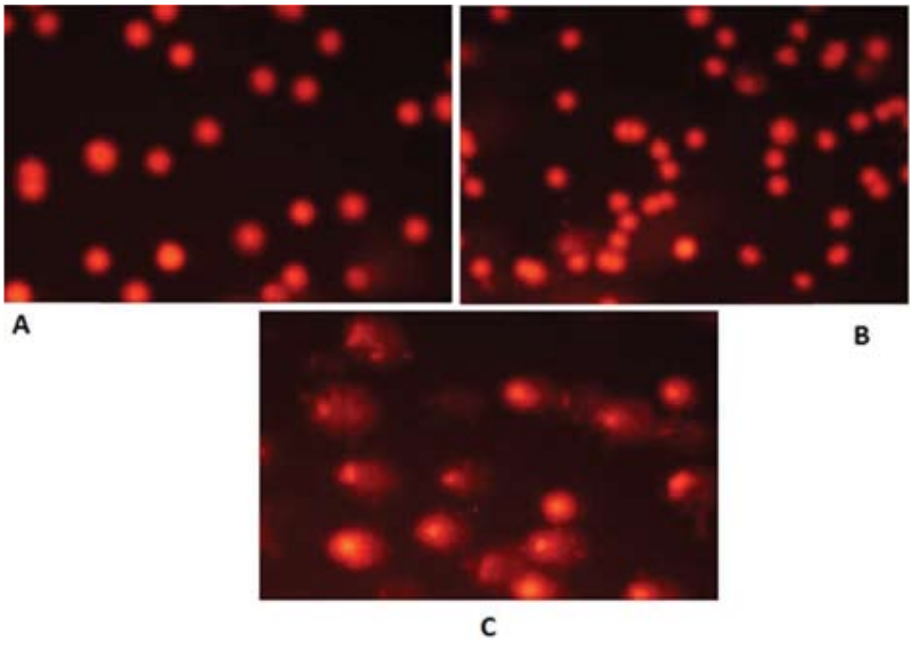

Fig 2. Photographic image of example of comet assay under fluorescence microscopy (x20 magnification). **a-** Group I (control), **b-** Group II (single injection group), **c-** Group III (double injection group)

Şekil 2. Floresans mikroskop altında (x20) komet yöntemi örneğinin fotoğrafik görseli. **a-** Grup I (Kontrol), **b-** Grup II (Tek enjeksiyon grubu), **c-** Grup III (Çift enjeksiyon grubu)

administration (Group II) whereas two consecutive injections of aglepristone (Group III) showed high genotoxic and cytotoxic effects on bone marrow in mid-gestation pregnancy termination in rabbits. No such differences were found between rabbits in control rabbits (Group I) and those treated once with aglepristone (Group II). Additionally, the comet assay results showed the single injection of aglepristone did not induce any DNA damage in bone marrow cells but two injections group increased the DNA damage according to OTM, tail % DNA and tail length frequencies significantly. Finally, comet assay results from blood samples did not show any significant difference between all groups.

The efficiency of aglepristone had been investigated in many species but no serious side effects or transient adverse effects after applications were reported [1-9]. The prominent side effects were noted as a short non-receptive period and irregular mating behavior after abortion, short time decrease in food consumption and the tendency of increase of some hematologic parameters during abortion duration in rabbits [7-9]. Such side effects had also been reported in dogs and cats but the reason of these unwanted effects remains unclear [4,5,25]. No information is available about the genotoxicity of aglepristone *in vitro* or *in vivo*. Other important antiprogestins are mifepristone, onapristone and lilopristone which exhibit a high chemical similarity with aglepristone. No clastogenicity and no genotoxic effects of these anti-progesterone compounds had been found [21,22]. There is no information on mifepristone; on the other hand lilopristone and onapristone have been shown to be inactive in gene mutation tests [26]. In this sense, the current study provides an important contribution to the literature with investigating potential genotoxic and cytotoxic profile of aglepristone.

Micronucleated erythrocytes could be spontaneously produced in peripheral blood in mammals but the frequency of erythrocytes might increase when they meet any genotoxic compounds [13]. Bone marrow samples are used for MN tests in rabbits, as micronucleated erythrocytes are removed by spleen from peripheral blood [19]. In our study two consecutive injection of aglepristone showed increased frequency of micronucleus in bone marrow of

rabbits. The elevation in the incidence of MNPCE in bone marrow results from chromosome damage in a short period of time and this allow us to evaluate short time exposure to chemicals [27]. It was also reported that binding affinity of the compounds could vary between species so it was suggested that the effects of antiprogestins must vary depending on receptor expression, the affinity to the receptor and the dose applied [10]. This could be connected with our results; high genotoxic, cytotoxic effects and DNA damage on bone marrow of two aglepristone applications. But the only one application of aglepristone cannot be effectively used in treatment of any pathologic case or pregnancy termination. General usage for termination the pregnancy in rabbits is double administration of aglepristone (10 mg/kg each dose) for a final concentration of 20 mg/kg. One of the limitations of our study is being not to investigate a dose of 20 mg/kg of aglepristone as a single dose. We choose a two consecutive administration in accordance with clinical use.

Aglepristone is recommended to use twice subcutaneous injection with a 24 h interval precisely. In the therapy of some pathologic cases such as pyometra, fibroadenomatous hyperplasia, more than two applications are used to cure effectively [2,3,19]. So we cannot recommend one application of aglepristone in order to minimize the genotoxic, cytotoxic effects and DNA damage. Uncontrolled reactive oxygen species (ROS) which is generated as a by-product of normal mitochondrial activity causes severe DNA damage response in aerobic cells. Cellular responses are described as the alterations in the cellular redox state during hypoxia or oxidative stress [28]. Superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) are important intracellular antioxidants and they are used as markers for detecting severity of oxidative stress [29,30]. These important markers were measured by our working group in mid-pregnancy termination in rabbits previously [31]. Marked increase of MDA after two injections of aglepristone were found and this result was statistically significant from one injection of aglepristone and control group. On the other hand contrary decrease had been detected in SOD and GSH in both aglepristone groups which are statistically significant compared to control group. The detected decrease in GSH after two injections of aglepristone was also significant from other two groups [31]. According to these results we speculated that the application of aglepristone on two consecutive days in abort induction could both trigger oxidative stress and change antioxidant enzyme activities in rabbits. Therefore, as an extension to our previous study, in the current study, aglepristone induced DNA damage on bone marrow of two injection group of rabbits and this was not seen in peripheral blood cells [31]. High level of ROS in a cell could increase the level of DNA damage [32]. We could also explain aglepristone induced DNA damage via the increased ROS level. The contradictory results between blood and bone marrow comet assays are interesting. We already know that the genotoxicity in blood cells reflects long term exposure to clastogenic chemicals unlike the bone marrow genotoxicity situation [13]. It has also been suggested in the report of Vasquez [33] that the *in vivo* comet assay from different tissues may show varied results. Thus aglepristone induced DNA damage in bone marrow cells may reflect short term effects with double dose treatment.

In conclusion the present study show the feasibility of bone marrow MN and comet assays in rabbits to determine the potential genotoxicity of aglepristone administered for abortion. The use of the aglepristone for abortion in small animals proved promising at single lower doses, however, long-term studies would be required to understand the carcinogenic effects for this compound.

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