Adipose tissue and liver in DMBA experimental intoxication

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Abstract
The present study aims to evaluate and verify the potential acute hepatic toxic effects of oral administration of 20 mg/kg/day, three times a week for four weeks, the polycyclic aromatic hydrocarbon, DMBA, an experimental model with rats, strain Wistar, male, eight weeks of age. At the end of the experimental protocol, all animals were sacrificed and necropsied. Liver and adipose tissue fragments were collected for histological evaluation and extraction and quantitative determination of the presence of DMBA by HPLC technique, in order to relate the toxicity of DMBA morphological changes in the liver and in the presence of DMBA liver and adipose tissues. In this study, all rats in which DMBA was administered showed a thinness compared to the control group were observed and mild to moderate pathological changes of hepatotoxicity. Chromatographic analysis quantified the presence of DMBA in adipose tissue of rat in the control group. This study of DMBA administration has shown a slight acute hepatic toxic effects by causing changes in the different parameters evaluated, which although not very significant, go against studies that point to the hepatotoxicity of DMBA.

Keywords: Rat, liver, polycyclicaromatichydrocarbons (PAH), 7,12-dimethylbenzantracene (DMBA).

INTRODUCTION
The location in the gastrointestinal tract of the liver facilitates the maintenance of internal homeostasis of an organism. The venous blood coming from the stomach and intestines passes through the portal vein into the liver and then enters the systemic circulation. Thus, the liver is the first organ to come into contact with nutrients, vitamins, minerals, chemicals and toxic environment, as well as, with the metabolic products of bacteria that enter the blood through the portal system. The efficient liver processes uptake remove these compounds absorbed into the metabolism, storage or biliary excretion[1]. The main functions of the liver can be changed by exposure to toxic substances. The liver cells exposed to significant concentrations of these substances, can undergo changes that lead to liver dysfunction without the occurrence of a significant cell damage, loss of function by cell damage and to failure of the body when chronic exposure causes replacement of normal tissue by tissue scar[2]. Although the liver is, quantitatively, the most important organ in the biotransformation of xenobiotics, other body tissues are also able to biotransform xenobiotics. Particularly active sites include the skin, lungs, kidneys and gastrointestinal tract. The ability of the liver and other extrahepatic tissues biotransformation xenobiotics, to inactive metabolites, less active or more active before reaching the bloodstream, is called pre-systemic effect of first passage[2][3].

Xenobiotics have varying degrees of water solubility and lipid solubility. The water-soluble are polar and ionizable substances in physiological pH values of the various compartments of the body, which cross biological membranes with great difficulty, being present in the glomerular filtrate and being easily excreted. The fat-soluble are non-polar and non-ionizable substances in the various compartments of the body, crossing quickly and effectively through biological membranes, especially by passive diffusion. Although they are filtered in the kidney glomeruli, they are readily reabsorbed by the renal tubules, the resorptive phase urine formation, returning to the systemic circulation. The same chemical property which enhances the bioavailability of such substances, can also hinder their renal excretion, since the clearance by the kidney requires that becomes more polar and hydrophilic, so that they can be dissolved in aqueous urine and excreted. Therefore, soluble xenobiotic need to undergo a biotransformation process for producing polar and hydrophilic compounds, which can be excreted via the bile or kidney. This process is mainly responsible for blood and tissue concentration of all chemical compounds that have been introduced into the body. Thus, the absorbed xenobiotic can be disposed without biotransformation or in the form of biotransformed metabolites[3]. In carrying out its functions, hepatocytes contain a lot of enzymes with oxidative and reducible functions, among which are the cytochrome protein.
450 system (P-450), flavin monooxygenases, peroxidases, hydroxylases, esterases and amidas. Other enzymes also present are glicor-initransfereases, sulfatransferases, methylases, acetyltransferases and bio-ferases. The enzyme systems involved in the interaction reactions of xenobi-tes are located in several compartments of the hepatocyte, as microsomes, mitochondria, cytosol, peroxisomes and lysosomes. All enzymes are of great importance in biotransformation of xenobi-tes, because these substances may change in several ways, rendering them beneficial, harmful or simply ineffective. In the case of drug, substance can change and for therapeutic purposes, the active drug may be converted into inactive drug or in an active or toxic metabolite, as well as, an inactive prod-ug can be converted to an active drug and a drug not excretable it can be con-verted to a metabolite capable of biliary excretion or renal route. When it comes to toxic substances, in some cases, its biotransformation converts them into less toxic or non-toxic easy excretion, acting liver detoxification function of the body. Sometimes, it results in production of a metabolite that is more toxic than the original compound, being named this process of bioactiva- tion. This undesirable side effect of biotransformation occurs when producing very reactive chemical species, usually electron- ic compounds with high affinity for the nucleophiles (DNA, proteins and lipids), which essentially modify cellular macromolecules, forming two or more aromatic rings benzene fused or grouped together and arranged in the linear or angular form, which confer different properties. In this particular case, the DMBA is comprising four aromatic rings arranged angularly form and two methyl groups. It is a PHA molecular weight 256,34 g/mol and a melting point of 122 to 123ºC. The chemical structure of DMBA derived from an- thracene (molecule with a linear arrangement), by adding two methyl groups to the L region and the addition of the benzene nucleus to the K region. This feature gives the molecule most carcinogenic effect.

In the literature we find the chemical name of the substance 7,12-dimethylbenzan-thracene, a variety of syn-onyms such as: dimethylbenzan-thracene; 7,12-di- methylbenzan-thracene; 7,12-dimethylbenz(a) an-thracene; 7,12-Dimethylbenzo(a)anthracene; 7,12-Di- methy1-1,2-benzanthracene; 7,12-Dimethy1-1,2-benz(a)anthracene; 7,12-Dimethy1-1,2-benzanthracene; 7,12-Dimethy1-1,2-benz(a)anthracene; 7,12-Dimethy1-1,2-benzanthracene; 7,12-Dimethy1-1,2-benz(a)anthracene; 7,12-Dimethy1-1,2-benzanthracene; 7,12-Dimethy1-1,2-benz(a)anthracene; 7,12-Dimethy1-1,2-benzanthracene. DMBA is a compound very slightly soluble in water, but is highly lipophilic, and therefore, rapidly absorbed and distributed in the body, can accumulate in adipose tissue. It is soluble in most organic sol- vents (toluene, chloroform, dichloromethane, etha- nol, diethyl ether, etc.).

In animal experiments, DMBA can be administered intravenously (IV), intraperitoneal (IP), subcuta-neous (SC), oral (gavage) and inhalation. Calculate the dose lethal to 50% of animals (LD50), the dose of the substance which produces the death of 50% of animals. The LD50 is 340 mg/kg orally in mice, 327 mg/kg orally in the rat and 54 mg/kg intrave-nously in rat [14], [15], [16], [18], [19], [20].

O DMBA na experimentação animal, pode ser ad-ministrado por via intravenosa (IV), intraperitoneal (IP), subcutânea (SC), oral (gastrostomia e inalató- ria. Calcula-se a dose letal para 50% dos animais (LD50), a dose da substância que produz a morte de 50% dos animais. A LD50 é de 340 mg/kg por via oral em murganhos, de 327 mg/kg por via oral na ratazana e de 54 mg/kg por via intravenosa em ratos [14], [15], [16], [18], [19], [20].

It has been awarded various toxicological, immuno-toxic, mutagenic, teratogenic and carcinogenic effects. Induces malignant transformation of cells in vitro transformation systems, with mutagenic for bacteria and mammalian cells. For all these effects, this chemical compound is used in medical and pharmaceutical research and has been the subject of many experimental studies. In the laboratory, the DMBA is used in animal testing in experimental models, such as an inductor and promoter malignant, regardless of the route of administration. It is described as a powerful complete carcinogen, because it acts both in the initiation and promotion, which explains its wide use in the study of chemical oncogenesis, as it ensures the development of the whole process without the need for other auxiliary substances [14], [16], [18], [21], [22].

The biotransformation of DMBA and other polycyclic hydrocarbons is an essential step in the beginning of carcinogenesis. The conversion of DMBA in its final carcinogenic metabolites is achieved primarily by enzymes of the cytochrome P450 family 1 (CYP). In particular, CYP1A isoforms are responsible for its bioactivation in the liver, the main body of DMBA metabolism, whereas CYP1B enzymes exert their activity primarily in extrahepa-tic tissues, such as the mammary gland. The liver plays a central role in the production of mutagenic agents which may be transported to other tissues, where they can also be metabolically activated to reactive metabolites with DNA. The toxic metabo-lites of DMBA include epoxides-diol that are capable of binding to DNA adenine residues, causing chromosomal damage, inducing tumors in various target tissues [20], [24].

In the liver, several studies have shown that DMBA induces toxic and carcinogenic lesions. In a study with rats it was intraperitoneally administered a single dose of 40 mg/kg body weight DMBA, were found hepatocellular damage, including structural disorganization, cirrhosis of the liver, congestion of blood vessels, intercellular hemorrhage, pyknotic nuclei of hepatocytes, activation Kupffer cells and sinusoidal dilation of blood. In the extracellular matrix was observed excessive deposition of collagen around the venous blood capillaries, characteristic of fibrosis and cirrhosis. These changes are con-sistent with those found for many other investiga-tors and are attributed to the increased production of reactive oxygen species and the inhibition or disruption to production of anti-oxidant enzymes. DMBA produces a much higher concentration of free radicals than non-carcinogenic compounds. These free radicals can result in cross-linked DNA, proteins and lipids from oxidative injury or different functional groups of important macromolecules, causing molecular and cellular damage. Fur-thermore, the toxic metabolites of DMBA including epoxide-diol in hepatic tissue are able to bind to DNA adenine residues, causing chromosomal damage. In 2011, Jesus made an experimental study with Wistar rats that used half the dose mentioned above (20 mg/kg three times a week for four we eks) and also found liver histopathological changes related to the DMBA [15], [16], [20].
Based on the grounds, sought to evaluate the re-
producibility of acute liver toxicity effects of DMBA
administration by gavage at a dose 20 mg/kg
DMBA, three times a week, for the histopathologi-
cal analysis of the samples taken from the liver of
rat subject the experimental protocol and quantifi-
cation of DMBA in liver and adipose tissue by High
Pressure Liquid Chromatography (HPLC).

MATERIAL AND METHODS
In this study it was used 18 healthy animals of the
species Rattus norvegicus, Wistar strain, male, with
an average age of 8 weeks. They randomly formed
two groups of nine animals each. The first group
 corresponds to the control group (C) which consis-
ted of animals maintained without any experimental
manipulation, which was not administered the toxic
(DMBA). Group II corresponded to the Test Group
(T), which consisted of animals submitted during the
trial period the administration by gavage of 20
mg/kg DMBA dissolved in oil, three times a week
(mondays, wednesday and friday) for four weeks.
In both groups the animals were observed daily and
weighed weekly in Contolo group and three times a
week in the test group.

Tissues collected at necropsy was done the routine
histological study of the liver and adipose tissue.
Were fixed in 10% buffered formalin and subjected
to histological processing using a 20 hour program
in the automatic tissue processor (Shandon Citadel
1000). The staining technique was used to routine
hematoxylin and eosin staining Mayer (HE). Also
liver and peri-renal fat fragments were collected
for frozen at -20°C for extraction and quantitative
determination of the presence of DMBA and its
metabolites through the technique of high pres-
sure liquid chromatography (HPLC). It is a liquid-
liquid extraction, which involves the partition of
the analyte between two immiscible liquid phases,
being used solvents, chloroform (CHCl
3) and me-
thanol (CH
3OH) in the proportion of two parts to
one. All extractions to hydrophilic compounds is
used PBS (phosphate buffer solution) as a solvent,
while for lipophilic compound is used a chloroform/
methanol solution (2:1). Due to the lipophilic nature
of DMBA was used as solvent chloroform/methanol
solution (2:1) [56]. This procedure begins by prepa-
ing the solvent and stirring in narrow mouth glass
bottle, 50 ml of chloroform and 25 ml of methanol.
For each sample is removed a quantity of 2 ml (ori-
ignal volume), which is placed in a glass test tube
and capped with parafilm until use.

Made up weighing the fragment frozen at -20°C
and placed in the hand held mixer tube type Cruscher,
where by means of vacuum caused by downward
movements and rotary piston, faded gradually. Was
added to one third of the 2 ml solvent and homoge-
nized again. The homogenate was poured into a
new tube with the corresponding number. This step
was repeated until the total volume of used solvent
(three times).

The tubes of each sample homogenate was cen-
trifuged at maximum speed for 10 minutes. At the
end, with a micropipette 2 µl the supernatant was
removed and the lower phase to two 2ml Eppen-
dorf tubes. Was measured final volume in each
 repercussions of these volumes may be changed depending on the
existing amount of lower phase). Once occurred
centrifugation, to remove the eppendorf tubes to a
suitable support and placed in freezer until HPLC
performed. This procedure was based on internal
laboratory methods.

These solutions were analyzed by high pressure
liquid chromatography (HPLC). This analysis was

RESULTS
In the control group and the test group, all animals
survived until the end of the experience, having
been performed euthanasia and necropsy of all
animals who started the experimental protocol (n
= 18). Rats from both groups were observed daily
for his health, well-being and possible pathologi-
cal, which does not registering significant changes.
Were also recorded the weights, performed weekly
in the control group and three times a week in the
test group during the experimental protocol and sa-
crificial.

Verified to a body weight gain significant (104,06 ±
17,46 g) in all rat of the control group (C01). How-
ever, there was a decrease in body weight at sacri-
fice (Figure 1).

Figure 1. Change in body weight of the control
group rats throughout the experimental time.

In the test group (T01), there was a variation in
the weights of the rats did not follow all the same
trend line (Figure 2). Most rats showed oscillations
in gain and loss weight body, resulting in a weight
gain little significant. The exception was the rat 13,
which over the experimental protocol until the 10th
administration, which matches the 1st administra-
tion of the 4th week, showed a progressive weight
gain, more significant between the 9th and the 10th
administration, followed by marked weight loss until
sacrifice. The rats 10,11 and 12 despite not having
had a significant weight gain until the 10th admi-
nistration, also showed a weight gain very signifi-
cant between the 9th and the 10th administration,
making losing weight marked by the sacrifice. Rats
12 and 15 were the only ones to sacrifice showed
body weights lower than those of the first weighing.
Since there was a decrease in weight to sacrifice in the control group, can not be compared to the 1st weighing values with the values of sacrifice and it was decided to make a difference between the first weight and the weight recorded in the last administration, to determine the gain or loss of body weight (Figure 3). In both groups, verified a gain of body weight, which was more significant in the control group, with an average gain of 104.06 ± 17.46 g. In the test group, this gain was only 19.84 ± 19.94 g, which may be related to the toxic effects of DMBA.

The presence of possible extreme values of the weights of organs was analyzed by Grubbs test using GraphPad Software (2004). When there are outliers in the data set, the hypothesis is null (H0) and when there is at least an extreme value, is the alternative hypothesis (Ha). The extreme values can be considered as manifestations of random variability inherent in data, pathological changes or just an error in the recording of sample data. This test is based on the sample numerical values (statistics), which is compared with a critical value based on the theory of random samples to decide whether or not a note considered extreme value. For each organ Z tabulated critical value (relative to a level of 0.05 significance, for n = 9) was equal to 2.2150. For the interpretation of the test results revealed that in the control group, the rat 8 had a high extreme value of the left lung. This is one of two rats (rat 8 and 9) that were identified macroscopic changes in the lungs. Also in this group, the rat 5 presents an extremely low value of the left kidney. For other organs accepted the null hypothesis. In the test group, the right lung was detected a high extreme value in the rat 14 and other organs also took up the null hypothesis. In the case of heart and spleen, was also carried out the measurement in three dimensions, length (a), width (b) and thickness (c) to calculate the volume through the formula las in the volume of a cone and volume an ellipsoid, respectively. Verified that the average volume of the test group of organs (Heart: 1561.90 ± 708.30 and Spleen: 4162.08 ± 1129.71) was slightly lower than in the control group (Heart: 1877.26 ± 230.74 and Spleen: 5741.97 ± 1508.78).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control Group</th>
<th>Test Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1.25 ± 0.06</td>
<td>3.09 ± 0.16</td>
</tr>
<tr>
<td>A2</td>
<td>1.29 ± 0.26</td>
<td>2.28 ± 0.20</td>
</tr>
<tr>
<td>A3</td>
<td>1.34 ± 0.32</td>
<td>1.36 ± 0.13</td>
</tr>
<tr>
<td>A4</td>
<td>1.33 ± 0.18</td>
<td>1.34 ± 0.09</td>
</tr>
<tr>
<td>A5</td>
<td>1.36 ± 0.44</td>
<td>1.26 ± 0.44</td>
</tr>
<tr>
<td>A6</td>
<td>1.37 ± 0.68</td>
<td>2.21 ± 0.68</td>
</tr>
<tr>
<td>A7</td>
<td>2.18 ± 0.64</td>
<td>2.58 ± 0.91</td>
</tr>
<tr>
<td>B7</td>
<td>1.89 ± 0.86</td>
<td>1.98 ± 0.61</td>
</tr>
<tr>
<td>D9</td>
<td>1.48 ± 0.32</td>
<td>1.72 ± 0.32</td>
</tr>
<tr>
<td>P4WD</td>
<td>2.05 ± 1.06</td>
<td>2.03 ± 1.00</td>
</tr>
<tr>
<td>P4WE</td>
<td>1.50 ± 0.30</td>
<td>1.25 ± 0.39</td>
</tr>
</tbody>
</table>

The Liver Weight Index is calculated by dividing the weight of the respective organ by body weight, at the same time (the sacrifice). Verified that there was no significant differences of the indexes in both groups, although the test group to give a slightly higher average (0.037 ± 0.004) than the control group (0.035 ± 0.004). The histological study carried out for liver tissue of animals belonging to the control group showed no changes with signified pathological market, being in agreement with that expected, in that this group of rats not administered the toxic and were maintained during the experimental protocol without manipulation (Figures 5, 6 and 7).
Figure 5. Histological section of liver from a rat of the control group, with a classic liver lobe outlined. Polyhedral structure with the central vein (V) which radiate hepatocytes and the port spaces (EP) to the periphery (HE, 5x the original).

Figure 6. Histological section of liver from a rat of the control group, with centrilobular hepatocytes fairly uniform and arranged in trabeculae. Predominately hepatocytes with a single round nucleus, with one (1) or two (2) prominent nucleoli and some binucleated hepatocytes (3). Visible Kupffer cells (4) (HE, 20x of the original).

Figure 7. Histological section of port space or portal triad of a rat of the control group. Branch of the hepatic artery (HA), portal vein branch (PV) and bile duct (DB) (HE, 20x of the original).

The liver tissue of rats belonging to the test group show slight histopathological changes, characterized by increasing the space between the trabeculae in periportal zones and center-lobular, hepatocytes with very basophil nuclei, degeneration of the area’s hepatocytes centrilobular and cloudy swelling of hepatocytes (Figures 8, 9, 10 and 11). These changes are hepatotoxicity signals.

Figure 8. Histological section of the liver of a rat of the test group, with increased space between trabecular and respective disorganization of hepatocytes in the periportal and centrilobular, compatible with edema (HE, 5x the original).

Figure 9. Histological section of the liver of a rat of the test group, with increased space between trabecular and hepatocytes with very basophil nuclei (HE, 10x of the original).

Figure 10. Histological section of the liver of a rat of the test group with degeneration of centrilobular zone. Hepatocytes with pale aspect and without defined contours (HE, 20x of the original).
It was also possible to quantify DNA in adipose tissue of four rats in the control group and five rats from the test group. In the chromatograms of extractions of hepatic tissue from these rats, peak retention time relating to DMBA (tr ≈ 29 min), there appeared in the rats in the control group (Figure 15), being detected in all the rats of the test group (Figure 16). The DMBA concentrations determined by HPLC in the adipose tissue of these rats are comprised between 2.75 x 10^{-3} and 6.04 x 10^{-3} mg DMBA mg per 1 g of adipose tissue.

DISCUSSION AND CONCLUSION

The study of DMBA oral administration of 20 mg/kg/day, three times a week for four weeks in an experimental model in rats (Rattus norvegicus), Wistar strain, males, 8 weeks old, showed have a slight acute liver toxicological effect. All mice survived until the end of the experiment, which leads to the conclusion that the dose is not lethal to this animal model. In literature, there are described studies that used a single intraperitoneal dose of 40 mg/kg body weight DMBA, which caused a more pronounced liver toxicological effect phenomena and chronic liver injury [25]. These results are corroborated by the weighing along the experimental protocol and sacrifice. Verified to a body weight gain in both groups, which was more significant in the control group compared to a gain very little significant in the test group. By analyzing the body condition of the rats of both groups, it is concluded that the rats from the test group have a greater body weight gain than the control group.
group showed a thinness compared to the control group. Also on weighing the organs, the mean weight in the test group was slightly lower than in the control group, having up verified in the same on heart and spleen volum. However, the average liver weight index a significant differences in the two groups, was slightly higher in the test group. This increase may be due to compensation for a liver regenerative process following the acute toxic effect of DMBA. In rats in which DMBA was administered were observed histopathological changes were mild to moderate hepatotoxicity. The analysis chromatographic quantified presence of DMBA in adipose tissue, although the percentage detected was less than 1% of dose delivered, which can be linked to the lipophilic character of DMBA, which makes it rapidly absorbed and distributed in the body. In liver tissue, DMBA was only quantified in only one mouse of the test group, which may be related to liver function in biotransform xenobiotics in their metabolites. Thus, the DMBA which in principle could be quantified in liver tissue, being biotransformed in their metabolites is deleted from this tissue. Thus, to carry out a complete study of the presence of this toxic in tissues, liver and fat, it must be identify and quantify also its metabolites. The lack of calibration curves for each metabolite, makes it impossible quantify them here and difficult its possible qualification, so a supplementary HPLC study is ongoing in the lab. The eventual analysis of excreted organic material (feces and urine) may also be a study of the complement of the presence of DMBA and its metabolites. In sum, we can say that the dose of DMBA administered causes changes in the different parameters evaluated, which although not very significant, show studies that point to the hepatotoxicity of the DMBA.

In continuation of this study now interests deepen this xenobioc accumulation in various organs of the body and relate this accumulation with some bodily functions and the general state of the organism.

It seems us important to realize if feeding may have some intervention in this toxic build up and how some diseases may increase or reduce their accumulation. Interests tell if some foods that have components that can stimulate or inhibit liver detoxification function may operate, the same with some drugs. Also the functional evaluation of certain organs such as the liver, can provide important data for the accumulation of this toxic.

One aspect that needs more research is the removal of DMBA in adipose tissue. Today there are theories that have as a starting point for explaining the accumulation of toxic diseases of the body. However there is a strong pressure for detoxification by taking products that are advertised as able to remove the toxic tissue and this is very important to study the conditions of removing toxic tissue and evaluate the possible consequences of this act. We propose to further study the use of animals divided into groups according to sex and age, even with different feeding conditions and subjected to chronic administration of some drugs. As the next step we tested products indicated to remove toxic body and monitor take these products to assay blood, urine, faeces and adipose tissue.

REFERENCES


