Effect of acrylamide on testis of albino rats

Ultrastructure and DNA cytometry study

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ABSTRACT

الأهداف: استكشاف التأثيرات الضارة للأكريلاميد على تركيب أنسجة الخصية في الجرذ الأبيض وذلك في محاولة لتوضيح المخاطر المحتملة على صحة الإنسان.

الطريقة: أجريت هذه الدراسة في قسم التشريح، كلية الطب، جامعة الملك عبدالعزيز، جدة، المملكة العربية السعودية خلال الفترة من ديسمبر 2010م إلى ديسمبر 2011م. شملت الدراسة 48 من جرذان ألبينو الذكور والبالغين وزن (300–250 غرام)، وقد تم تقسيمهم عشوائياً إلى 6 مجموعات. تم استخدام المجهر الإلكتروني والتقنيات النسيجية باستخدام صبغة الفولجين لإجراء فحص الأنسجة، بالإضافة إلى دراسة الحمض النووي الخلوي.

النتائج: أشارت نتائج الدراسة إلى أن إعطاء جرعة من الأكريلاميد (25 ملي غرام/ كيلوغرام/10أيام) قد أظهرت تأثيراً طفيفاً على الخصية سواء كان الأكريلاميد عن طريق الفم أو الحقن داخل الصفاق. وعلى الجانب الآخر فإن إعطاء الأكريلاميد بجرعة (50 مجم/ كيلوغرام/10أيام) قد أظهر نسبة ضرر عالية للخصية وخصوصاً مع الحقن داخل الصفاق مقارنةً بإعطاء الجرعة بالفم، حيث أدى ذلك إلى التالي: تدمير الخلايا التناسلية، والعديد من الخلايا العملاقة متعددة النواة مع وجود تجاويف وفراغات بين الخلايا التناسلية.

خاتمة: أظهر هذا البحث أخطار الأكريلاميد وتأثيره المحتمل على صحة الإنسان والتي ستكون أكثر وضوحاً مع فترة أطول من التعرض للأكريلاميد. ونحن نوصي بالإقلال من محتوى الأطعمة لهذه المادة ومراعاة الطرق المختلفة لخفض مستوى مادة الأكريلاميد أثناء تحضير الأطعمة المختلفة.

Objectives: To explore the harmful effects of acrylamide on the structure of testis in albino rats, in an attempt to clarify its potential risks on human health.

Methods: The present study was carried out in the Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia from December 2010 to December 2011. Forty-eight adult male albino rats (250-300 g) were divided randomly into 6 groups. Electron microscopy and histochemical techniques using Feulgen stain were used to conduct the morphological study. In addition, DNA cytometry method was used.

Results: Rats treated with acrylamide 25 mg/kg body weight for 10 days showed mild affection, whether acrylamide was administered orally or intraperitoneally. On the other hand, the testis of the group treated with a dose of 50 mg/kg/10 days showed damage, especially with intraperitoneal administration in comparison to oral treatment. This was in the form of degeneration of germ cells, numerous multinucleated giant cells with sloughed seminiferous epithelium, and vacuolation in-between the germ cells.

Conclusion: Exposure to acrylamide produced degenerative changes in the testis, which were more prominent with a longer period of exposure. Recommendations are necessary to decrease acrylamide level in different foods, and ways to decrease the acrylamide formation during preparation of different foods should be advertised.

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Acrylamide is a white crystalline odorless compound, soluble in alcohol and water, but insoluble in heptane and benzene. It is formed of "acrylic-amide" with its formula C_3H_5NO. Acrylamide exists in 2 forms; highly toxic monomer, and nontoxic polymer. Solid form is stable at room temperature, but may polymerize aggressively when melted, or exposed to oxidizing agents.[1] Average daily adult intake of acrylamide in most populations was estimated to be approximately 0.5 microg/kg body weight (BW).[2] However, intake may vary widely from 0.3-2 microg/kg BW/day, or may reach even 5 microg/kg BW/day. Certain carbohydrate-rich foods, particularly asparagines when reacting with sugar in high temperatures more than 200°C during cooking, it is where acrylamide is formed, and the reaction is named - Millard reaction.[3] It was found that when those foodstuffs are heated to temperatures exceeding 120°C, it yielded acrylamide concentrations up to one mg/kg in carbohydrate-rich foodstuffs, in addition, the authors added that foods prepared, or purchased in restaurants had concentrations up to almost 4 mg/kg.[4] The early findings tended to focus on starch-rich foods such as fried potatoes, French fries, and crisp-bread, all of which showed relatively high levels of acrylamide. Besides potatoes, particular cereals, coffee, and crisp-bread were considered as relevant sources of human exposure, since they are consumed on a regular basis by a broad group of consumers.[5] Moreover, acrylamide was assessed by the International Agency for Research on Cancer in 1994 as "probably carcinogenic to humans (IARC Group 2A)".[6] Based on the positive bioassay results in mice and rats, supported by evidence that acrylamide is biotransformed in mammalian tissues to genotoxic metabolite, the biotransformation process by which acrylamide is converted to glycidamide is possible in humans, and can be demonstrated to occur efficiently in both human and rodent tissues.[2,6] Effects of acrylamide on reproductive system of rats have included decreased sperm count, increased abnormal sperm morphology, severe testicular damages, such as vacuolation and swelling of the round spermatid, and break of DNA during specific germ cell stages.[7,8] In addition, male rats administered with acrylamide exhibited significant reductions in mating, fertility, as well as transport of sperm in uterus. Impaired fertility associated with effects on sperm count and sperm mobility parameters have been demonstrated in male rats exposed to 15 mg/kg BW/day, or more for 5 days. It was added that male mice treated with 3.5 mg/kg by gavage 2 times/week for 8 weeks showed testicular atrophy, degenerating spermatids and spermatocytes, also multinucleated giant cells were observed.[9,10] Therefore, the present study was designed in a dose-dependent manner to investigate the harmful effects of acrylamide on the structure of the testis in albino rats, in an attempt to clarify its potential risks on human health. In addition, to state which way of exposure (intraperitoneally [i.p] or oral) is more effective.

Methods. The study protocol was approved by the Hospital Biomedical and Research Ethics Committee, Faculty of Medicine, King Abdulaziz University, Kingdom of Saudi Arabia, and the procedures were carried out according to the Guide for the Care and Use of Laboratory Animals by National Institutes of Health.

Acrylamide. Acrylamide powder (99% purity) was obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). It was dissolved in saline and/or distilled water.

Animals care and use. The present study was carried out in the Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia from December 2010 to December 2011. Forty-eight adult male albino (Sprague-Dawley strain) rats, aged 85-90 days, and weighing 250-300 g were used in this study. Each rat was housed in one per cage, and maintained under a controlled environment with average temperature (20-27°C) throughout the experimental period, water and food availability, and standard light-dark cycle at the animal house.

Study design. After one week of acclimatization, the animals were divided randomly into 6 groups (n=8), and created as Group I and Group II. Group I was subdivided into 3 subgroups: Control group - received i.p saline injection daily for 10 days; Group Ia - received i.p injections of acrylamide in a dose of 25 mg/kg BW daily for 10 days;[11] Group Ib - received i.p injections of acrylamide in a dose of 50 mg/kg BW daily for 10 days.[12] Group II was subdivided into 3 subgroups: Control group - received fresh distilled water (by oral gavage) daily for 10 days; Group IIa - received 25 mg/kg BW acrylamide orally (by oral gavage) daily for 10 days;[13] Group IIb - received 50 mg/kg BW acrylamide orally (by oral gavage) daily for 10 days.[14]

Tissue sampling and processing. At the end of the experiment (10 days), all rats were anesthetized using ether inhalation. Specimens of testes were extracted and processed for electron microscopic examinations, samples approximately one mm^3 were obtained, and fixed in 2.5% glutaraldehyde, and processed to obtain Epon capsules. Then, semithin sections approximately one µm thickness were cut, and stained with toluidine blue, and examined using a light microscope. Ultrathin

sections (50-60 nm thick) were cut using an LKB ultramicrotome (Ultratome NOVA, LKB 2188, Bromma, Sweden), and stained, and examined by Philips 201 transmission electron microscope at 60-80 kv in Transmission Electron Microscope Unit (Philips Industries, Eind-hoven, The Netherlands). For light microscopic examination, samples approximately ½ cm^3 were taken from the mentioned organs, and fixed in 10% buffered neutral formalin processed to obtain paraffin blocks, then serial sections 5-6 µm thick of the testes were sliced, and stained with Feulgen stain.

DNA cytometry. Feulgen reaction. The Feulgen staining reaction specifically stains the DNA to give specific red-purple staining of the nuclear DNA. Cytoplasms showed pale or no staining. The stained DNA can then be quantitated when analyzed on the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England), (that is, after DNA staining, the nuclear-integrated optical density (OD) is the cytometric equivalent of its DNA content).[15]

Image analysis. The nuclear DNA analysis was performed at the Pathology Department, National Research Center, Cairo, Egypt according to the following steps: system calibration was carried out before each measurement session; and calibration slides provided with the system were used; the slides to be examined were placed on the stage of the microscope; and focused at high power magnification (X400). The light source is set to the required level, successful adjustment of illumination was checked-for on the video monitor.[15]

DNA analysis. The DNA content analysis of the basal compartment of the testis was performed on real time image from the microscope on the video monitor. The DNA ploidy analysis was performed in normal control specimen using the DNA cytometry software. Automatically nuclear boundaries were selected by the image analysis system. Only separate, intact nuclei were measured. Distorted or overlapping nuclei and nuclear fragments were manually eliminated from the measurement. All these facilities were supplied as editing function in the Leica Qwin 500 image analysis systems (LEICA Imaging Systems Ltd, Cambridge, England). The OD of the selected nuclei were measured and automatically converted by the system into the DNA content. The OD software was used for quantitative analysis of DNA reaction. Color intensity is proportional to DNA content in the nucleus. Results were displayed as a frequency histogram on the monitor generated by plotting the DNA content versus the number of nuclei counted. Then, scaling of the data was automatically performed by the system for optimal resolution, interpretation, and statistical assessment. Histograms should not be overly compressed so as to obscure rare events; neither should they be overly expanded so as to split the data into false, or nonexistent populations. The percentages of cells within each selected area, the DI (deviation index) and the CV (coefficient variation) for these populations were all calculated and determined automatically by the system. All collected data were stored to be reanalyzed.

Interpretation of DNA histograms. The DNA histograms were classified as diploid, tetraploid, and aneuploid based on the amount of DNA relative to the normal control.[16] The diploid position (2C) for the study population was determined after calibration of the system. The DNA histograms obtained by image analysis were classified as follows: diploid type - when a single peak is found in the diploid, or near diploid region with a DNA index ranging from 0.9-1.1, and fewer than 20% of the cells are present at the tetraploid position; tetraploid type - when there is a peak in the diploid region, and a second peak with more than 20% of the cells in the tetraploid region with a DNA index ranging from 1.8-2.2; and aneuploid type - when at least 10% of the total events show a distinct abnormal peak outside the 2C or 4C position. The proliferation index (PI) is automatically expressed as the percentage of cells engaged in the S-phase of the cell cycle. Previously, it was classified with slight modification into low (<10%), medium (10-20%), or high (>20%).[17-19]

Statistical analysis. The quantitative data were expressed as mean and standard deviations, while qualitative data were expressed as numbers and percentage as appropriate. Quantitative data from different groups were compared using one-way ANOVA followed by Bonferroni correction test, while qualitative parameters were compared using Chi-square test. Probability value $p<0.05$ was considered statistically significant. The statistical analysis was conducted at a 95% confidence level. All statistical analysis was performed with GraphPad InStat v3.10 software (GraphPad Software, Inc, San Diego, CA, USA).

Results. ***Histopathological observations.*** In the present study, the examined specimens of the testes of the control group showed sections of the seminiferous tubules containing numerous spermatozoa. These tubules were separated by intervening connective tissue containing Leydig cells. Close examination of the wall of the seminiferous tubules showed that it consisted of germinal epithelium supported by Sertoli cells. The germinal epithelium comprised different stages of the spermatogenic series namely; spermatogonia, spermatocytes, spermatids, and spermatozoa arranged

from without inward. The testis of the rats receiving 25 mg/kg/10 days, whether oral, or i.p administration showed some seminiferous tubules with normal appearance of the spermatogenic series and characteristic whorly appearance of the sperms inside the lumen. However, some of the seminiferous tubules had an irregular outline. Other seminiferous tubules showed depletion of germ cells, and slightly congested blood vessels were observed (**Figure 1a**). The histological sections of the testis of the rats receiving 50 mg/kg/10 days i.p showed that most of the seminiferous tubules suffered from marked depletion of the spermatogenic cells, many of which were seen sloughed in the lumen. Moreover, the striking histological change was the appearance of numerous multinucleated giant cells. These cells were large rounded cells with abundant eosinophilic cytoplasm and multiple peripherally arranged nuclei. They were located free in the lumen of the seminiferous tubule, in-between sloughed germ cells, and close to the limiting membrane. In addition, there was marked congestion of the blood vessels in-between the seminiferous tubules (**Figure 1b**). Detailed examination of the seminiferous tubules revealed formation of vacuoles of different sizes between spermatogenic cells that were resting over a wavy limiting membrane. Moreover, the multinucleated giant cells contained multiple nuclei of variable shape and size and their cytoplasm was vacuolated (**Figure 1c** and **1d**). The testis of the rats receiving 50 mg/kg/10 days orally showed similar histological features to that previously described group but to a lesser extent.

Ultrastructural observations. Control group. Electron microscopic examination revealed that the spermatogonia were resting on the limiting membrane, and their nuclei were characterized by their regular nuclear membrane and dispersed chromatin. The cytoplasm contained numerous rounded mitochondria with normal cristae (**Figure 2a**). The primary spermatocytes were identified by their rounded nucleus with clumps of dense chromatin and their pale cytoplasm. The cytoplasm was rich in mitochondria. Spermatids at different maturation stages were recognized by their rounded nuclei that were covered by the characteristic acrosomal cap. The mitochondria were peripherally arranged in the cytoplasm (**Figure 2b**). Furthermore, the

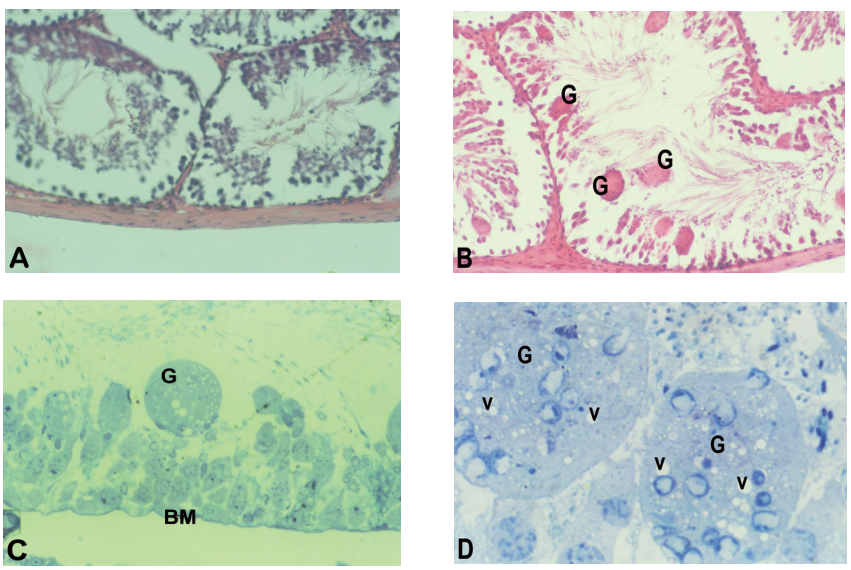

Figure 1 - Photomicrograph of rat testis section from: **A**) Group Ia (receiving 25 mg/kg/10 days/intraperitoneally [i.p]) showing depletion of germ cells in seminiferous tubules (Hematoxylin & Eosin [H&E] X200); **B**) Group Ib (receiving 50 mg/kg/10 days/i.p) showing depletion of spermatogenic cells. Note the numerous multinucleated giant cells (G) between the sloughed spermatogenic cells (H&E x200). Photomicrograph of a semithin rat testis section from: **C**) Group Ib (receiving 50 mg/kg/10 days/i.p) showing depletion and separation of the spermatogenic cells, multinucleated giant cells (G) and irregular limiting membrane (BM) (Toluidine blue X400); **D**) Group IIb (receiving 50 mg/kg/10 days/orally) showing 2 multinucleated giant cells (G) with large cytoplasmic masses containing multiple, oval, and rounded nuclei. Many vacuoles (V) were observed (Toluidine blue X1000).

nuclei of the supporting Sertoli cells were located close to the limiting membrane, and were typically pyramidal in shape with dispersed chromatin. Their cytoplasm contained mitochondria and lipids.

Effect of acrylamide on Group Ia receiving 25 mg/kg/10 days/i.p. On the ultrastructural level, some Sertoli cells contained myelin-like figures and lysosomal dense bodies (**Figure 3a**). Few primary spermatocytes showed vacuolation of the cytoplasm. Many spermatogonia appeared degenerated with vacuolated cytoplasm and mitochondria (**Figure 3b**). However, the spermatids appeared were apparently not affected.

Effect of acrylamide on group (IIa) receiving 25-mg/kg/10 days/orally. Electron microscopic examination demonstrated that some primary spermatocytes appeared normal, with the characteristic perinuclear chromatoid body (**Figure 3c**), while other primary spermatocytes showed irregular nuclear membrane, aggregated dense chromatin and dense cytoplasm. Moreover, no significant changes could be identified in the spermatids (**Figure 3d**).

Effect of acrylamide on Group Ib receiving 50-mg/kg/10 days/i.p. Ultrastructurally, the degenerated spermatogonia showed degenerated vacuolated cytoplasm (**Figure 4a**). Moreover, degenerated spermatocytes, with characteristic perinuclear chromatoid body, manifested vacuolated cytoplasm. In addition, many spermatids showed signs of degeneration in the form of vacuolation in the cytoplasm (**Figure 4b**). On the other hand, the degenerated Sertoli cells showed pyknotic-shrunken nuclei and severely vacuolated cytoplasm. Again, many ill-defined degenerated cells with irregular pyknotic nuclei were seen close to the limiting membrane (**Figure 4c**).

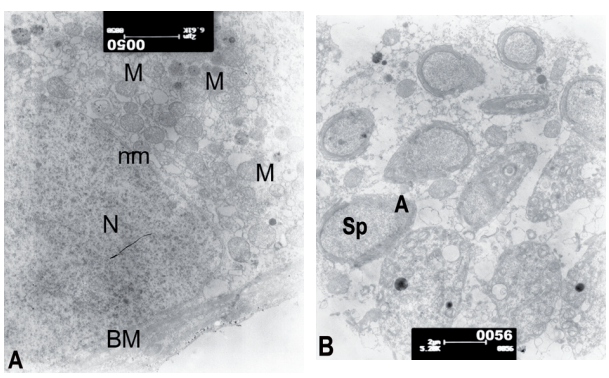

Figure 2 - Transmission electron micrograph of rat testis section from control group showing: **A**) part of a spermatogonium resting on the limiting membrane (BM) with numerous mitochondria (M) in the cytoplasm (Cy). Note the regular nuclear membrane (nm), and the dispersed chromatin of the nucleus (N) (X6600); **B**) early spermatid (Sp) with characteristic acrosomal cap (Ac), and peripherally arranged mitochondria (M) in the cytoplasm (X5200).

Effect of acrylamide on Group IIb receiving 50 mg/kg/10 days/orally. The ultrastructural changes affecting the testis of this group were comparable to those described for Group Ib. Degenerated spermatogonia and Sertoli cell manifested pyknotic nuclei and highly vacuolated cytoplasm. Moreover, some fields showed massive basal cells distortion with degenerated vacuolated cytoplasm, containing remnants of mitochondria and rough endoplasmic reticula, so that it was difficult to differentiate between cells whether spermatogonia or Sertoli cell (**Figure 4d**). Degenerated spermatocytes with irregular nuclei and dense chromatin were noticed (**Figure 4e**).

Apoptosis (Programmed cell death). In an attempt to clarify whether the above-mentioned light and electron microscopic changes in spermatogenic cells were due to necrosis or increased degree of apoptosis, sections obtained from various groups of tested animals were subjected to Feulgen stain and studied using image analyzer. The apoptotic cells were identified by being light pink in color in comparison to normal cells that showed a red-purple nucleus with dispersed chromatin remnants. Data analysis with the image analyzer revealed that in the control group, the mean number of apoptotic cells per 10 circular seminiferous tubules cross sections was found to be 5/10 apoptotic cells. With the administration of acrylamide at a dose of 25 mg/kg/10 days either orally or i.p, this number slightly increased to be 6/10 circular seminiferous tubules cross-sections. However, with a dose of 50 mg/kg/10days administered orally this number increased by 180% as compared to the control to be 9/10 circular seminiferous tubules cross-sections. An outstanding increase of the mean number of apoptotic cells by 300% occurred with the dose of 50 mg/kg/10 days administered i.p in comparison with the control to reach 15/10 circular seminiferous tubules cross sections (**Figures 5a & 5b**).

Study of DNA cytometry control group. Analysis of the data of control group demonstrated that most of the cells were diploid cells (2C) (57%) followed by triploid cells (3C) (22.8%) with high proliferation index, monoploid cells (<1.5c) were 19.3%, tetraploid cells (4C) were 0.88%, while no aneuploid (>5c) were found (**Table 1**) with significant difference between groups (p <0.0001) using Chi-square test (**Figure 6a**).

Group of testes of rats receiving 25 mg/kg/10 days whether oral or intraperitoneal. As shown in **Table 2**, testes of rats from group (receiving 25 mg/kg/10 days) whether orally or i.p) shows that most of the cells were

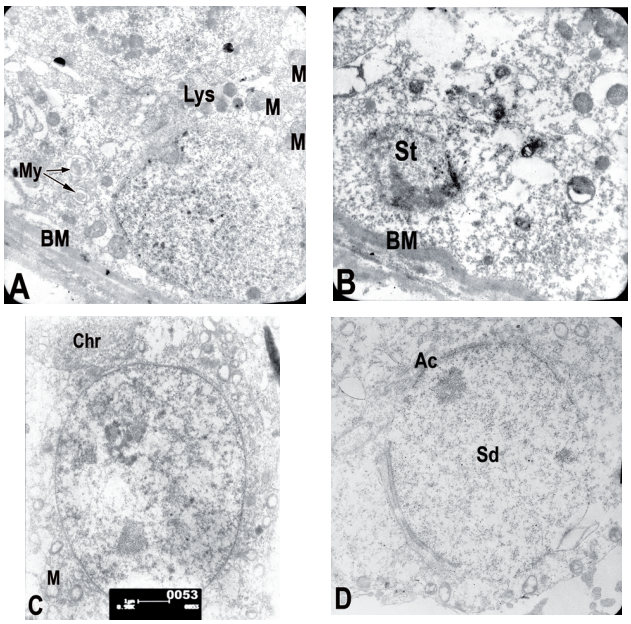

Figure 3 - Transmission electron micrograph of a rat testis section: **A**) Group Ia (receiving 25 mg/kg/10 days/ intraperitoneally [i.p]) showing Sertoli containing myelin-like figures (My), lysosomal dense bodies (Lys), and mitochondria (M). Notice the limiting membrane (BM) (X4000); **B**) Group Ia (receiving 25 mg/kg/10 days/i.p) illustrating part of a spermatogonia (Sg) resting on BM with vacuolated cytoplasm and M (X4000); **C**) Group IIa (receiving 25 mg/kg/10 days/ orally) showing part of the 1ry spermatocytes with the characteristic perinuclear chromatoid body (Chr). Note many M (X9000); **D**) Group IIa (receiving 25 mg/kg/10 days/orally) showing part of a spermatid (Sd) with typical acrosomal cap (Ac), clear nucleoplasm showing no significant changes (X9000).

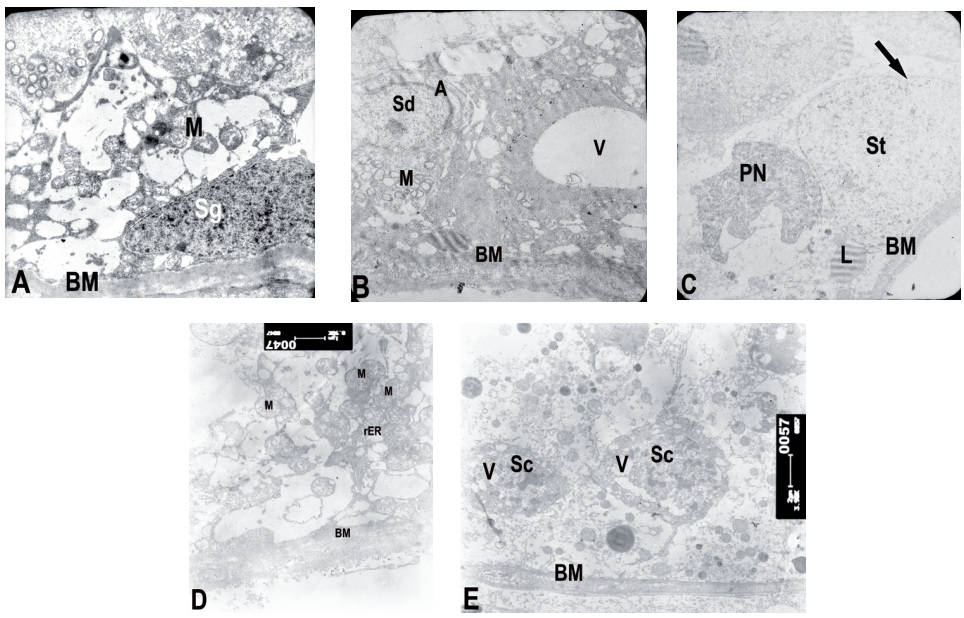

Figure 4 - Transmission electron micrograph of rat testis section: **A**) Group Ib (receiving 50 mg/kg/10 days/ intraperitoneally [i.p]) showing part of a spermatogonium (Sg) adjacent to the limiting membrane (BM) with severely degenerated vacuolated cytoplasm (X4000); **B**) Group Ib (receiving 50 mg/kg/10 days/i.p) showing severely vacuolated cytoplasm (V) of Sertoli cells adjacent to the BM, Spermatid (Sd) with degenerated cytoplasm. Note normal mitochondria (M) with short cristae. Notice the acrosomal cap (A) (X9000); **C**) Group Ib (receiving 50 mg/kg/10 days/i.p) showing a degenerated cell with a pyknotic shrunken nucleus (PN). Note Sertoli cell (St) with its characteristic indentation of the nucleus resting on the BM, and lipid droplet (L), and indentation of the nucleus (↑) (X9000); **D**) Group IIb (receiving 50 mg/kg/10 days/orally) showing cell distortion and degenerated vacuolated cytoplasm of basal cells possibly spermatogonium, or Sertoli cell since degeneration is massive, and cannot differentiate cells. Note remnants of rough endoplasmic reticulum (rER) and M (X9000); **e**) Group IIb (receiving 50 mg/ kg/10 days/orally) showing degenerated spermatocytes with irregular nuclei and dense chromatin. Note the vacuolated cytoplasm (V) (X4000).

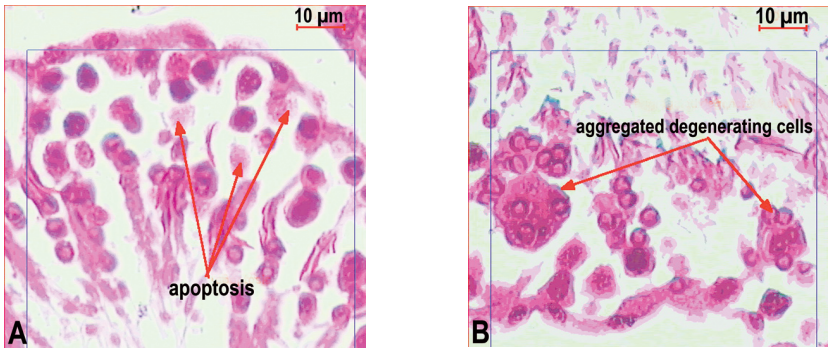

Figure 5 - Photomicrograph of rat testis section from group receiving 50 mg/kg/10 days/intraperitoneal showing: **A**) 3 apoptotic cells; and **B**) aggregated degenerated cells (Feulgen X400).

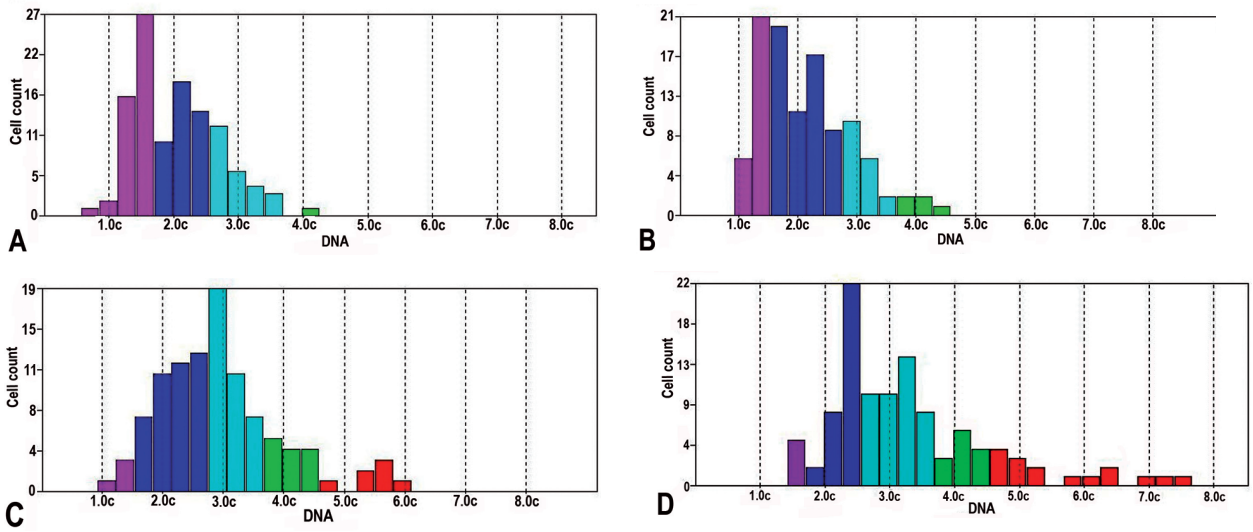

Figure 6 - A deoxyribonucleic acid (DNA) histograms showing: **A**) Control group; **B**) Group receiving 25 mg/kg/10 days whether oral or intraperitoneal; C) Group receiving 50 mg/kg/10 days/orally; D) Group receiving 50 mg/kg/10 days/intraperitoneally.

diploid (2c) (52.34%) with high proliferation index followed by triploid cells (3c) (21.50%), monoploid (<1.5c) (20.56%), tetraploid (4c) were 4.67 then cells with >4.5c were 0.94%, meanwhile no aneuploid cells were found with significant difference between groups (p<0.0001) using Chi-square test (**Figure 6b**).

Group of testes of rats receiving 50 mg/kg/10 days/orally. As shown in **Table 3**, DNA ploidy revealed that the highest number of cells with triploid cells (2.5c-3.5c) (46.15%) followed diploid (2C) (29.81%) with extremely high proliferation index, many tetraploid cells (4C) (14.42%), some aneuploid cells (5.77%), indicating increased mitotic index, very high proliferation and some cells with abnormal DNA content, which means positive result for malignancy; cells >4.5c were 6.73%, cells <1.5c were 2.89% with significant difference between groups (p<0.0001) using Chi-square test (**Figure 6c**).

Group of testes of rats receiving 50mg/kg/10 days/intraperitoneal. As shown in **Table 4**, DNA ploidy showed that triploid cells were 37.04% followed by diploid (2C) (29.6%) with very high proliferation index; tetraploid (4C) (18.5%) and many aneuploid cells (9.3%), Indicating very high proliferation with many cells with abnormal DNA content that means highly positive result for malignancy with significant difference between groups (p<0.0001) using Chi-square test (**Figure 6d**).

Statistical analysis. As shown in **Tables 5 & 6**, comparing the groups regarding all cells (total number of cells), there no significant difference between control and group receiving 25 mg/kg/10 days (p>0.05), but there is significant high increase in all cells in the group receiving 50 mg/kg/10 days orally than control (p=0.0001). Moreover, there is a significant higher increase in all cells in the group receiving 50 mg/kg/10 days i.p than control (p=0.0001).

Comparing the groups regarding the aneuploid cells (5cER), there no aneuploid cells in the control and group receiving 25 mg/kg/10 days, but the aneuploid cells started to appear with the group receiving 50 mg/kg/10 days orally, but increased in numbers with group receiving 50 mg/kg/10 days i.p.

Comparing the groups regarding the diploid cells (1.5-2.5c), there insignificant increase in groups receiving 25 mg/kg/10 days, and 50 mg/kg/10 days orally than control but there is significant increase in group receiving 50 mg/kg/10 days i.p than control (p=0.0001).

Comparing the groups regarding the diploid cells (2.5-3.5c), there significant increase in groups receiving 25 mg/kg/10 days and 50 mg/kg/10 days orally and 50 mg/kg/10 days i.p comparing to the control (p=0.0001).

Discussion. The findings presented here add to our knowledge of the histopathological alterations induced by treatment with acrylamide in the testes. In the present work, the testis of the rats that received 50 mg/kg/10 days showed marked depletion of the spermatogenic cells in most of the seminiferous tubules as compared to the control rats. Moreover, an explicit aggregation of multinucleated giant cells with multiple peripherally arranged nuclei was identified. In addition, marked congestion of the blood vessels were noticed in-between the seminiferous tubules. Scientists explained the appearance of multinucleated giant cells in the seminiferous tubules of rats subjected to acrylamide by the inability of primary spermatocytes to undergo meiotic divisions to generate haploid sperm cells, thus, the additional DNA replication gives rise to multinucleated giant cells.[10] A previous study[3] used acrylamide in a dose of 50 mg/kg/10 days observed numerous multinucleated giant cells and sloughed seminiferous epithelium.

In the present study, numerous vacuoles were observed in-between the spermatogenic cells. Similarly, scientists observed vacuolation in the seminiferous tubules after acrylamide treatment. The impact of these insults on the seminiferous tubules was reflected in the decreased sperm count.[20] Others also mentioned increased abnormal sperm morphology.[21] Accordingly, it could be postulated that if acrylamide caused this insult in seminiferous tubules of rats such impact could occur in humans and result in fertility problems.

Table 1 - The deoxyribonucleic acid (DNA) ploidy and ploidy related-parameters for the control group.

Range	Number of cells	(%)	DNA index	Mean ± SD	*P*-value
All	114	(100)	1.00	2.07 ± 0.66	0.0001
<1.5c	22	(19.3)	0.62	1.27 ± 0.16	
1.5c-2.5c	65	(57.0)	0.94	1.95 ± 0.32	
2.5c-3.5c	26	(22.8)	1.43	2.96 ± 0.29	
3.5c-4.5c	1	(0.9)	1.96	4.05 ± 0.00	
>4.5c	0	(0.0)	-	-	
5cER (>5.0c)	0	(0.0)	-	-	

5cER - aneuploid cells having DNA content exceeding 5c

Table 2 - The deoxyribonucleic acid (DNA) ploidy and ploidy related-parameters of testis of rats from group receiving 25 mg/kg/10 days whether oral or intraperitoneal.

Range	Number of cells	(%)	DNA index	Mean ± SD	*P*-value
All	107	(100.0)	1.00	2.15 ± 0.76	0.0001
<1.5c	22	(20.6)	0.60	1.29 ± 0.15	
1.5c-2.5c	56	(52.3)	0.91	1.96 ± 0.32	
2.5c-3.5c	23	(21.5)	1.37	2.94 ± 0.26	
3.5c-4.5c	5	(4.7)	1.82	3.91 ± 0.15	
>4.5c	1	(0.9)	2.12	4.54 ± 0.00	
5cER (>5.0c)	0	(0.0)	-	-	-

5cER - aneuploid cells having DNA content exceeding 5c

Table 3 - The deoxyribonucleic acid (DNA) ploidy and ploidy related-parameters of testis of rats from group receiving 50 mg/kg/10 days/orally.

Range	Number of cells	(%)	DNA index	Mean ± SD	*P*-value
All	110	(100.0)	1.38	2.95 ± 1.01	0.0001
<1.5c	3	(2.7)	0.61	1.31 ± 0.14	
1.5c-2.5c	31	(28.2)	0.94	2.02 ± 0.26	
2.5c-3.5c	48	(43.6)	1.38	2.95 ± 0.28	
3.5c-4.5c	15	(13.6)	1.87	4.02 ± 0.30	
>4.5c	7	(6.4)	2.56	5.49 ± 0.43	
5cER (>5.0c)	6	(5.5)	2.62	5.62 ± 0.30	

5cER - aneuploid cells having DNA content exceeding 5c

Table 4 - The DNA ploidy and ploidy related-parameters of testis of rats from group receiving 50 mg/kg/10 days/intraperitoneally.

Range	Number of cells	(%)	DNA index	Mean ± SD	*P*-value
All	108	(100.0)	1.60	3.31 ± 1.25	0.0001
<1.5c	0	(0.0)	-	-	-
1.5c-2.5c	32	(27.1)	1.05	2.18 ± 0.30	
2.5c-3.5c	40	(33.9)	1.43	2.95 ± 0.30	
3.5c-4.5c	20	(16.9)	1.90	3.94 ± 0.31	
>4.5c	16	(13.6)	2.74	5.67 ± 1.00	
5cER (>5.0c)	10	(8.5)	3.01	6.23 ± 0.85	

5cER - aneuploid cells having DNA content exceeding 5c

The histological findings were confirmed by the ultrastructure examination of the seminiferous tubules. It was noticed that, with increasing dosages of acrylamide both Sertoli cells and spermatogenic series suffered from degenerative changes in the form of vacuolation of the cytoplasm, degeneration of the mitochondria with lost cristae and pyknosis of nuclei which were more marked when the dose was 50 mg/kg/10 days. In male toads, it is mentioned that administration of acrylamide resulted in necrosis of the seminiferous tubules with varying degrees of vacuolation of the mitochondria.[22] Moreover, it is observed nuclear vacuolation of germ cells in testes of adult mice subjected to a dose of 100-150 mg/kg acrylamide.[23]

In the present investigation, apoptosis (programmed cell death) was studied in the seminiferous tubules in order to evaluate whether the depletion of the spermatogenic cells in response to acrylamide administration occurred merely due to degenerative changes or it was accompanied by an increase in the apoptotic process. Data analysis with the image analyzer revealed that the mean number of apoptotic cells increased by 180-300% in animals receiving 50 mg/kg/10 days orally and i.p respectively compared to the control rats. Such findings correlate with the described affection of the spermatogenic cells and reflect cellular stress due to acrylamide administration. In that respect, it is reported the presence of significant increase in the number of apoptotic cells in seminiferous tubules of testes isolated from acrylamide-treated rats.[3] In addition, acrylamide-treated rat showed alteration of the genes related to the function of testis, apoptosis, cellular redox, cell growth, cell cycle, and nucleic acid binding.[10]

As cells respond to DNA damage by inducing apoptosis, DNA cytometry was conducted in the present investigation and revealed the occurrence of a significant degree of aneuploidy in animals receiving oral and intraperitoneal 50 mg/kg/10 days of acrylamide. Such finding demonstrate that the damaging effect of acrylamide on the rat testis reached beyond an inflammatory process to produce severe changes in the cells indicative of high proliferation with many cells with abnormal DNA content that reflects a highly positive result for malignancy. Such abnormal DNA content was in agreement with previous findings, which observed chromosomal aberrations, unscheduled DNA synthesis and DNA breakage of germ cells in male rats exposed to acrylamide.[14,24] Moreover, it is reported that cell proliferation and cell cycle delay were found in spermatocytes by acrylamide treatment.[10] In addition, acrylamide disturbs the genes related to cell proliferation and cell cycle, which might result in abnormal histopathological features in reproductive organs.[3] It is worth mentioning at this point that workers exposed to acrylamide in monomer and polymer production plants had double the risk of pancreatic cancer, 75% excess incidence of brain cancer, 15% increase in lung cancer and 9% increase in all cancers combined.[25]

In conclusion, exposure to acrylamide produced degenerative changes in the testis, which were more

Table 5 - Results of acrylamide effects at different doses, expressed as mean and standard deviation (mean ± SD).

Range	Control		25 mg/kg/10 days		50 mg/kg/10 days oral		50 mg/kg/10 days intraperitoneal	
	Number of cells	Mean ± SD	Number of cells	Mean ± SD	Number of cells	Mean ± SD	Number of cells	Mean ± SD
All cells	114	2.05 ± 0.68	107	2.13 ± 0.83	104	3.09 ± 1.13	108	3.42 ± 1.26
<1.5c	22	1.3 ± 0.16	22	1.3 ± 0.15	3	1.3 ± 0.14	0	-
1.5c-2.5c	65	1.9 ± 0.32	56	2.0 ± 0.32	31	2.0 ± 0.26	32	2.2 ± 0.30
2.5c-3.5c	26	1.4 ± 0.29	23	2.9 ± 0.26	48	3 ± 0.28	40	3 ± 0.30
3.5c-4.5c	1	-	5	3.9 ± 0.15	15	4 ± 0.30	20	3.9 ± 0.31
>4.5c	0	-	1	-	7	5.5 ± 0.43	16	5.7 ± 0.99
5cER	0	-	0	-	6	5.6 ± 0.3	10	6.2 ± 0.85

Table 6 - Comparison of deoxyribonucleic acid (DNA) aneuploidy results of the control and exposed animals regarding all cells and diploid cells.

Groups	*P*-value all cells	95% confidence interval	*P*-value 1.5c-2.5c	95% confidence interval	*P*-value 2.5c-3.5c	95% confidence interval
Control versus 25 mg/kg/10 days	0.541	(0.34 - 0.34)	0.167	(0.25 - 0.05)	0.0001	(1.72 - 1.28)
Control versus 50 mg/kg/10 days, oral	0.0001	(1.24 - 0.56)	0.184	(0.28 - 0.08)	0.0001	(1.79 - 1.41)
Control versus 50 mg/kg/10 days IP	0.0001	(1.54 - 0.86)	0.0001	(0.48 -0.12)	0.0001	(1.79 - 1.41)

$p<0.05$ was considered as statistically significant different from the control, IP - intraperitoneal

prominent with the longer period of exposure. The present study pointed out the hazards of acrylamide and its possible effect on human health, and proved that the intraperitoneal route is more dangerous than oral route. The present study expands the available information concerning the hazards carried by the consumption of acrylamide on testis. Although the doses of acrylamide utilized in the present investigation were higher than the average dietary daily intake in humans, 0.4-5 microg/kg body weight/day,[26] yet the cumulative effects of such toxicant on human health are still waiting to be fully identified. The work proved that further studies focusing on the influence of acrylamide on different organs in smaller doses for prolonged periods could aid in the full understanding of hazards implicated by this substance.

Recommendations are necessary to decrease acrylamide level in different foods and ways to decrease acrylamide formation during preparation of the different foods should be advertised. Also further studies concerning cumulative effects of acrylamide exposure are highly needed.

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References

1. Schuur A. Health impact assessment of policy measures for chemicals in non-food consumer products. Available from URL: http:// www.rivm.nl/bibliotheek/rapporten/320015001.pdf
2. Rice JM. The carcinogenicity of acrylamide. ***Mutat Res*** 2005; 580: 3-20.
3. Yang HJ, Lee SH, Jin Y, Choi JH, Han DU, Chae C, et al. Toxicological effects of acrylamide on rat testicular gene expression profile. ***Reprod Toxicol*** 2005; 19: 527-534.
4. Sharp D. Acrylamide in food. ***Lancet*** 2003; 361: 361-362.
5. Stadler RH, Scholz G. Acrylamide: an update on current knowledge in analysis, levels in food, mechanisms of formation, and potential strategies of control. ***Nutrition Reviews*** 2004; 62: 449-467.
6. Pelucchi C, La Vecchia C, Bosetti C, Boyle P, Boffetta P. Exposure to acrylamide and human cancer--a review and meta-analysis of epidemiologic studies. ***Ann Oncol*** 2011; 22: 1487-1499.
7. Weiss G. Cancer risks. Acrylamide in food: uncharted territory. ***Science*** 2002; 297: 27.
8. Richmond P, Borrow R. Acrylamide in food. ***Lancet*** 2003; 361: 361-362.
9. Taeymans D, Wood J, Ashby P, Blank I, Studer A, Stadler RH, et al. A review of acrylamide: an industry perspective on research, analysis, formation, and control. ***Crit Rev Food Sci Nutr*** 2004; 44: 323-347.
10. Yang HJ, Lee SH, Jin Y, Choi JH, Han CH, Lee MH. Genotoxicity and toxicological effects of acrylamide on reproductive system in male rats. ***J Vet Sci*** 2005; 6: 103-109.
11. Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. ***J Agric Food Chem*** 2002; 50: 4998-5006.
12. Tyl RW, Friedman MA. Effects of acrylamide on rodent reproductive performance. ***Reprod Toxicol*** 2003; 17: 1-13.
13. Nordin-Andersson M, Walum E, Kjellstrand P, Forsby A. Acrylamide-induced effects on general and neurospecific cellular functions during exposure and recovery. ***Cell Biol Toxicol*** 2003; 19: 43-51.
14. Paulsson B, Grawé J, Törnqvist M. Hemoglobin adducts and micronucleus frequencies in mouse and rat after acrylamide or N-methylolacrylamide treatment. ***Mutat Res*** 2002; 516: 101-111.
15. El-Sharkawy SL, Abbas NF, EL-Hefnawy NG. Prognostic Markers in Prostatic Carcinoma. ***Methods of Cancer Diagnosis, Therapy and Prognosis: General Methods and Overviews, Lung Carcinoma and Prostate Carcinoma*** 2009; 2: 465.
16. Mi LJ, Patil J, Hornbuckle WE, Cote PJ, Gerin JL, Tennant BC, et al. DNA ploidy analysis of hepatic preneoplastic and neoplastic lesions in woodchucks experimentally infected with woodchuck hepatitis virus. ***Hepatology*** 1994; 20: 21-29.
17. Lauwers GY, Grant LD, Scott GV, Carr NJ, Sobin LH. Spindle cell squamous carcinoma of the esophagus: analysis of ploidy and tumor proliferative activity in a series of 13 cases. ***Hum Pathol*** 1998; 29: 863-868.
18. Trere D, Zilbering A, Dittus D, Kim P, Ginsberg PC, Daskal I. AgNOR quantity in needle biopsy specimens of prostatic adenocarcinomas: correlation with proliferation state, Gleason score, clinical stage, and DNA content. ***Clin Mol Pathol*** 1996; 49: 209-213.
19. Salim EI, Omar KM, Abou-Hattab HA, Abou-Zaid FA. Pituitary toxicity but lack of rat colon carcinogenicity of a DC-magnetic field in a medium-term bioassay. ***Asian Pac J Cancer Prev*** 2008; 9: 131-140.
20. Oishi S. Effects of propyl paraben on the male reproductive system. ***Food Chem Toxicol*** 2002; 40: 1807-1813.
21. Abramsson-Zetterberg L. The dose-response relationship at very low doses of acrylamide is linear in the flow cytometer-based mouse micronucleus assay. ***Mutat Res*** 2003; 535: 215-222.
22. Friedman M. Chemistry, biochemistry, and safety of acrylamide. A review. ***J Agric Food Chem*** 2003; 51: 4504-4526.
23. Shipp A, Lawrence G, Gentry R, McDonald T, Bartow H, Bounds J, et al. Acrylamide: review of toxicity data and dose-response analyses for cancer and non-cancer effects. ***Crit Rev Toxicol*** 2006; 36: 481-608.
24. Sega GA, Generoso EE. Measurement of DNA breakage in specific germ-cell stages of male mice exposed to acrylamide, using an alkaline-elution procedure. ***Mutat Res*** 1990; 242: 79-87.
25. World Health Organization. Health Implications of Acrylamide in Food. Geneva (CH): WHO Headquarters: 2002.
26. Kornbrust BA, Stringer MA, Lange NEK, Hendriksen HV. Asparaginase - An Enzyme for Acrylamide Reduction in Food Products. In: Whitehurst RJ, van Oort M, editors. Enzymes in Food Technology. 2nd ed. Oxford (UK): Wiley-Blackwell Publishing Ltd; 2009. p. 59-87.