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Research Article

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The impact of some Bisphenol-A based restorative materials on the estrogen receptors activity

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ABSTRACT

Recently concerns were raised about the adverse effect of the leached monomers, from the resin-based materials, particularly bisphenol-A (BPA) because of its demonstrated estrogenic effect. Three commercially available bisphenol-A glycidyl methacrylate (Bis-GMA) resin-based restorative materials were used in this study. These products were two pit and fissure sealants Fisseal and Clinpro™. and one resin composite restorative materials Filtek™ Z 350XT flowable restorative. The extracted immersion media were analyzed using HPLC for the detection of the leached out BPA. The leached components were administered intragastrically daily to the test groups and the equivalent volumes of the immersion media were administered to the control groups of mice for one week, two weeks and three months. At the end of each experimental period, blood samples were withdrawn from retro-orbital plexus of all mice. The collected serum samples were tested for the activity of estrogen receptor using Mouse Estrogen Receptor Alpha (ERα) ELISA kit. Then all animals were sacrificed and ovaries from females as well as testes from male mice were dissected and fixed in formalin saline 10% for histological examination. The results revealed that flowable composite immersed in citric acid showed higher BPA monomer release and higher values of estrogen receptor activity than both sealants. Higher values of estrogen receptor activity were observed for female mice and citric acid subgroups. Ovaries of female mice were more affected than testes of males and these affections ranged from increased proliferation of the interstitial stromal cells in the medulla to severe congested ovarian blood vessels.

Keywords: Pit and fissure sealant, Flowable composite, HPLC, Estrogen receptors, Bisphenol-A.

INTRODUCTION

Over the past 20 years, the use of resin-based restorative materials in clinical dentistry had a major influence on esthetic dentistry. Bisphenol-A glycidyl methacrylate (Bis-GMA) is one of the components used in resin-based dental composites. This component is known to remain in the polymer after the curing process is completed. Furthermore, residual unpolymerized oligomers have been reported to leach out into the oral environment **[1, 2].**

Besides to a slow and persistent degradation of dental composites in the oral cavity caused by chemicals and mechanical factors, recent researches demonstrated that human saliva exhibited cholesterol esterase hydrolase activity that had a catalytic effect leading to the breakdown of these residual Bis-GMA components in dental resin composites **[3, 4]**. Moreover, these leached components may have an estrogenic effect that might contribute to the inadvertent exposure of patient to xenoestrogens. These are a group of chemicals that exerts a biological reaction

comparable to that of natural estrogen, which involves molecules possessing a double benzoic ring. In dentistry, such molecules include bisphenol-A therefore, potential bisphenol-A sources in dental materials mainly include sealants, composites, adhesives and polycarbonates esthetic brackets **[5, 6]**.

Many studies have found that the exposure of humans and animals to BPA induces hormonal related impacts on obesity, nervous system, thyroid function, breast, prostate and development of cancer and others [**7**-**16].** Although resin-based restorative materials available on the market fulfill Environmental Protection Agency Standards regarding the BPA content; nonetheless, these standards are based on the toxic effects of BPA rather than on their estrogenic effects.

Since the detection of the estrogenic activity of resin based composites and sealants by Olea group in 1996 **[17],** some researchers have turned their attention to quantify the amount of leached BPA from dental resin-based restorative materials using different analytical methods and techniques. BPA, its dimethacrylate derivatives, Bisphenol-A diglycidylether, Bis-GMA, and ethoxylate and propoxylate of Bisphenol-A was detected by HPLC analysis from Bis-GMA based resin composites and sealants in concentrations greater than the minimum amount to show estrogenicity **[18, 19].** However, another *in vitro* study failed to detect any leachable amounts of BPA from seven dental sealants, although other compounds were detected **[20]**. Moreover, BPA was detected in some saliva samples at one and three hours after sealant placement. While, no BPA was detected beyond three hours in saliva samples or in any of the serum samples. Furthermore, the BPA concentration was found to be proportional to the amount of sealant placed **[21]**. Thus, the evidence remains inconclusive owing to the lack of consistency in results in different *in vitro* and *in vivo* studies. To further investigate the estrogenic effect of BPA content of these materials, this study was performed to assess the estrogenic effect of leached BPA on male and female mice and its impact on the reproductive organs of male and female mice after different administration period.

EXPERMENTAL SECTION

Resin-based materials

Three commercially available Bis-GMA resin-based restorative materials, two pit and fissure sealants Fisseal and Clinpro™ and one flowable composite restorative material Filtek ™ Z350XT flowable restorative were used in this study. Table (1) illustrates the investigated resin-based restorative materials.

Table (1): The investigated resin-based restorative materials

Commercial name	symbol	Type	Composition	Manufacturer
Fisseal	C1	Pit and fissure sealant	BisGMA, TEGDMA	Promedica, Germany Lot no. (1236506)
Clinpro™ Sealant	C2	Pit and fissure sealant	BisGMA, TEGDMA	3M ESPE Dental Products, USA Lot no. (12636)
Filtek™ Z350XT flowable Restorative	C3	Flowable nano- composite	BisGMA, TEGDMA, BisEMA	3M ESPE Dental Products, USA
			Zirconia/silica and silica; Nanoparticles 55vol%-65wt%	

Monomer:

Bisphenol-A (HPLC standard): 4,4'-isopropylideneddiphenol: 97% pure Aldrich- Chemie, Albuch, Germany.

Preparation of leached components from resin based materials:

Two hundred and ten resin-based disc shaped specimens were prepared (n= 70 specimens for each material). The discs were 15 mm in diameter and 0.5 mm in thickness according to ISO 4049: 1988 Dentistry-resin-based filling materials **[22]** for sorption and solubility testing, with the exception of storage time and media used. Teflon mold was used to produce the specimens in the required shape and size. The investigated materials were packed in to the teflon mold and built up in one increment. Then the packed materials were topped by a glass slide to flatten the specimen surface. The specimens were light cured with Halogen curing unit (Chromalux E; Mega Physik Dental, Germany, light intensity of 440 m W/ cm^2). The light curing tip was placed at the top surface of each specimen and then light-cured for 40s to simulate the clinical situation, according to the manufacturer's recommendations.

Preparation of the immersion media

Two immersion media namely artificial saliva and citric acid were used for the extraction of BPA from the investigated resin-based materials in the current study. The artificial saliva was prepared by dissolving 0.4 g/L NaCl, 0.4 g/L KCl, 0.795 g/L $CaCl_2 \cdot 2H_2O$, 0.69 g/L $NaH_2PO_4 \cdot H_2O$, 0.005 g/L $Na_2S \cdot 9H_2O$, and 1.0 g/L CO $(NH_2)_2$in one liter of distilled water **[23].** The pH of artificial saliva was adjusted to be 6.8 with KOH using pH meter (Hanna,

PH 211, Micro processor, PH Meter, USA) . The second immersion media was citric acid with pH = 4 prepared by mixing 614.5 ml, 0.1 M Citric acid ($C_6H_8O_7$), and 385.5ml, 0.2 M disodium phosphate (Na_2HPO_4) **[24]** .

After curing, the specimens of each resin-based material were divided into two subgroups according to the type of immersion media (n=35) and immediately immersed in 5 mL of the prepared respective media. All specimens were subjected to thermocycling in a thermal cycling simulation machine (Biometra, T3 thermocycler, Germany) in distilled water bath between 5 °C and 55 °C with 30-s dwell times for 5000 cycle. The samples were stored in a dark box at 37°C temperature. Five samples from each subgroup were involved in this study. The immersion medium was renewed daily for one week, then weekly for three months. The extracted immersion media were analyzed using HPLC (High Performance Liquid Chromatography) for the detection of the leached bisphenol-A.

Detection of the leached BPA from resin-based materials

Bisphenol-A was determined by HPLC, Agilent technologies 1100 series, equipped with a quaternary pump (G131A model), according to the method described by *Al-Hiaysat et al* [25].

The standard preparation was performed by dissolving 30 mg of BPA standard (Aldrich- Chemie, Albuch, Germany) in one ml mobile phase. Serial dilutions were prepared from the standard; 10 µl from serial dilution of standard were injected in HPLC to draw standard curve with different concentration.

The extracted media (20µl) was injected in HPLC. Three measurements were determined for each sample and the means were calculated. The value of leached BPA was calculated from the standard curve µg/ml. Separation was achieved on octadecylsilane (ODS) reversed phase column (C18, 25×0.46 cm i.d., 5 µm) and UV detector was set at 280 nm. All determinations were conducted at room temperature using mobile phase which consisted of A and B, A was acetonitrile /water (50/50) V/V and B was acetonitrile. A and B were filtered through a 0.45 µm membrane filter (Satorious, Goettingen, Germany) and degassed prior to use. A linear gradient from solvent A to solvent B over 15 min (0–100%) and a flow rate of 1 ml min-1 was applied [11].

Experimental design (*in vivo* study)

A total number of 240 mice, aged 40 days, 120 female and 120 male were used in this study. They were housed in the Animal House Unit of the National Research Centre (NRC), Cairo, Egypt at controlled temperature of 24 ± 1°C on a 12 h light/12 h dark cycle. Food (standard laboratory rodent chow diet) and water were supplied *ad libitum.* The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre.

Animals grouping

The animals were randomly divided into four main groups, each contains 60 mice (30female and 30 male) one control group and three test groups. The three test group of mice received the leached components of material (C1), (C2) and (C3) respectively. Each main group was subdivided into two subgroups of 30 mice (15 females and 15 males) according to the immersing media (artificial saliva or citric acid). Each animal received 5 ml of the eluted substances orally using animal feeding intubation.

Each subgroup was further subdivided into three groups of 10 mice (5 males and 5 females), the first group received the leached component daily for one week, the second received the leached component daily for two weeks and the third received the leached component daily for three months. In order to standardize the animal handling and any stresses created during the intra-gastric administration, the control group received equivalent volumes of the two media using the same technique for the same administration periods.

At the end of each experimental period, blood samples were withdrawn from retro-orbital plexus of all mice. Sera were separated from the blood samples and stored at -70°C pending for analysis of estrogen receptor activity.

Determination of mouse estrogen receptor alpha (ERα) activity

The activity of estrogen receptor alpha (ERα) was quantified in serum by enzyme linked immunosorbent assay (ELISA) technique using mouse estrogen receptor alpha assay kit (Glory Science, U.S.A)

Histological examination

After collection of blood samples, all animals were sacrificed and ovary from females as well as testis from males were dissected and fixed in formalin saline 10% for histological examination. Paraffin bees wax tissue blocks were prepared for sectioning at 4µ thickness by sliding microtome (Leitz-3002, Germany). The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain for routine investigation then examination was done through the light electric microscope (Lap. Photo, Nikon 236033, Japan)

Statistical analysis

Data were presented as mean and standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Data showed normal (parametric) distribution; so regression model using repeated measures Analysis of Variance (ANOVA) was used in testing significance for the effect of resin composite, immersion media, immersion time and their interactions on mean bisphenol-A monomer release. Repeated measures Analysis of Variance (ANOVA) was used also in testing significance for the effect of resin composite, storage media, gender, storage time and their interactions on mean estrogen release. Tukey's post-hoc test was used for pair-wise comparisons between the groups when ANOVA test is significant. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with SPSS version 20.

RESULTS AND DISCUSSION

Although resin-based restorative materials were introduced for use in anterior regions where esthetics are of prime concern, the continuous improvement in their chemistry and properties expanded their use to almost all cavities, whether anterior or posterior. In addition, resin-based materials are also used as pit and fissure sealants for caries prevention. The effectiveness of these sealants in caries prevention promoted their use to the extent that their application became obligatory in some countries **[26]**. However, concerns were raised about the adverse effect of the leached monomers, from these resin-based materials, particularly BPA because of its demonstrated estrogenic effect **[17]**. All these data together with the limited available information about the extent of the cumulative estrogenic effect on both male and female made it crucial to quantify BPA release and determine its impact on estrogen receptors activity.

The aim of the current study was to quantitatively assess the effect of the different pH and time of exposure on the amount of leached BPA monomer from two pit and fissure sealants and one flowable composite. Our aim was also extended to elucidate the impact of leached BPA monomer on the activity of estrogen receptors of both male and female mice over three month.

The result revealed that flowable composite showed higher mean BPA monomer release than both sealants as shown in **Fig. (1)**. These results are in agreement with ***Olea et al.*** **[17]** .This may be attributed to the fact that the amount of leached monomers from any polymeric material varies inversely with the degree of conversion. Since the filler volume fraction in flowable composites is relatively higher than sealants, and since these fillers may act as scattering centers, flowable composites may exhibit lower translucency compared to sealants. Such reduced translucency may decrease the degree of conversion of flowable composites leading to higher BPA release **[27].**

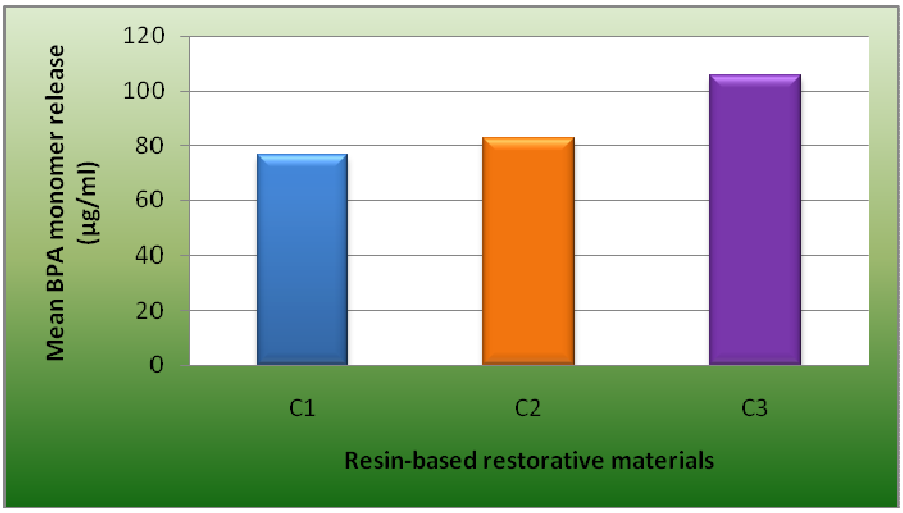

Fig. (1): Comparison between BPA monomer release (µg/ml) for the different resin-based restorative materials

Table (2): Comparison between estrogen receptors activity in ng/ml of resin-based restorative materials regardless of other variables

C1		C2		C3		*P*-value
Mean	SD	Mean	SD	Mean	SD	
1.08 [c]	0.14	1.15 [b]	0.16	1.22 [a]	0.20	0.002*

**: Significant at P ≤ 0.05, Different superscripts are statistically significantly different according to Tukey's test.*

These BPA release profiles were also reflected on the estrogen receptors activity results where the flowable composite showed the higher mean estrogen receptors activity than the two pit and fissure sealants as shown in **Table (2).**

The relation between BPA release and the estrogen receptor activity may be due to the similarity of the phenol groups on both BPA and estradiol. This resemblance allows the BPA molecule to trigger estrogenic pathways by activation of estrogen receptors **[28, 29]**. These results are in conformity with *Schafer et al.* **[30]** who demonstrated that BPA can increase breast cancer cells proliferation, that are known to proliferate under estrogenic stimulation, It must be noted that such effects were reported to occur only at concentrations 100,000 times higher relative to estradiol. These finding can also be related to those reported by *Trami et al.* **[31]** who investigated two pit and fissure sealants and found that both were estrogenic although none of them contained BPA. This is despite the fact that BPA-DMA, which is also estrogenic, was found to be included in these two sealants in an amount greater than the minimum amount to show estrogenicity. It must be taken into consideration this latter study investigated these sealants without thermocycling while temperature was found to play an important role in the conversion of the BPA-DMA to the more estrogenic form BPA **[31]**.

The results of the current study showed that citric acid had statistically significant higher mean BPA monomer release than artificial saliva as shown in **Fig. (2).**

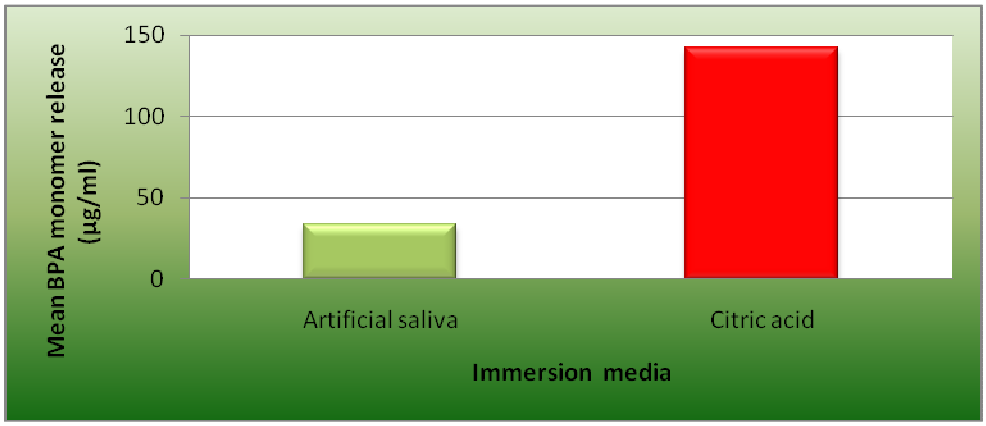

Fig. (2): Comparison between BPA monomer release (µg/ml) of immersion media

In accordance of our results, *Göpferich* **[32]** indicated that acidic media had a significant impact on the amount of leachable components of the resin as these media accelerate the hydrolysis of the ester bond linking the BPA molecules to the resin-based materials. This may lead to entrance of acids inside the polymer bulk, which may result in swelling. The intrusion of acids triggers further chemical degradation which results in the formation of oligomers and then monomers. The progressive degradation process changes the microstructure of the material through the formation of pores, *via* which oligomers, residual monomers, degradation products and additives are released. Alongside, the pH inside the pores begins to be controlled by degradation products. Finally, oligomers and monomers are released, leading to erosion with subsequent weight loss of the polymer **[33]** .These results were also reflected on the estrogen receptor activation where the citric acid group showed significantly higher mean estrogen receptor activity than artificial saliva as shown in **Table (3).**

Table (3): Comparison between estrogen receptors activation (ng/ml) of immersion media regardless of other variables

Artificial saliva		**Citric acid**		***P*-value**
Mean	**SD**	**Mean**	**SD**	
1.11	0.16	1.19	0.19	0.010*

**: Significant at P ≤ 0.05*

This may be explained by the fact that BPA leached under extreme pH by Bis-GMA based composites and sealants exceed the minimum amount to show estrogenicity **[34]**.

The present results showed that BPA release was detected in all groups till day six after which all groups didn't show any monomer release. Even during these six days, the release significantly decreased over time as shown in **Fig. (3).**

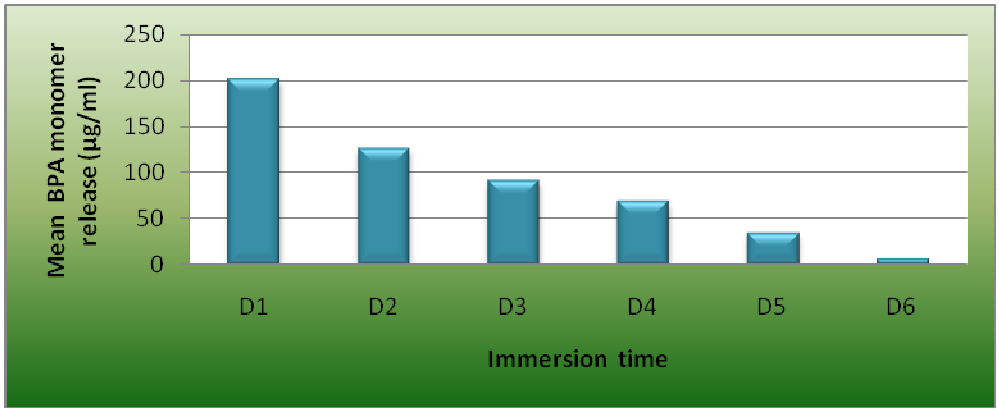

Fig. (3): Comparison between BPA monomer release (µg/ml) at different immersion periods.

These results are in agreement with *Michelsen et al.* **[35],** who found that BPA was leached out from all tested composite and sealant samples and this release decreased with time. It has been shown that the majority of the leachable organic components eluted from composite resin materials within the first seven days after curing. *Ferracane and Condon* **[3]** reported that most of the unbound organic components were eluted within 24 h after curing. However, these results are contradictory with those reported by *Wada et al.* **[36]**, who indicated that HPLC failed to detect BPA in any of the elutes of 24 commercially available flowable composites. This difference may be attributed to the fact that this latter study used water as immersion medium without thermocycling. As previously mentioned, thermocycling has a direct effect on the ester bond breakage that account for the BPA release.

The highest estrogen receptor activity was found to occur after one week and two weeks study period as shown in **Table (4).**

Table (4): Comparison between estrogen receptors activation (ng/ml) at the different administration periods regardless of other variables

1 week		2 weeks		3 months		*P*-value
Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
1.23 [a]	0.15	1.21 [a]	0.15	1.02 [b]	0.14	0.010*

**: Significant at P ≤ 0.05, Different superscripts are statistically significantly different*

After three months, there was a statistically significant decrease in mean estrogen receptor activity. This finding shows a close relevance to the amount of release of BPA from these materials which stopped after seven days. The decrease in the estrogen receptor activity after the initial high activation may be due to the fact that BPA is rapidly metabolized in liver *via* conjugation of glucuronic acid with BPA to form the metabolite (BPAG) **[37]**. Although the release was found to stop after about one week, the activated estrogen receptors remained relatively high till after two weeks and this represents the time needed for metabolic clearance of the administered BPA. These results support the fact that persistent estrogen receptor activation needs exposure to significant amounts of BPA which must be continuous and *via* multiple sources **[38]**.

The present results demonstrated that, females experienced higher mean estrogen receptor activation than males as shown in **Fig. (4).**

Regarding the effect on female mice, the current results are relevant with those reported by *Al-Hiyasat et al.* **[25],** who indicated that BPA significantly affected female mouse fertility due to the activation of estrogen receptors by BPA. The estrogenic effect of BPA is markedly affected by the concentration of estrogenic receptors thus female were more sensitive to BPA than males. Also, BPA is soluble in lipid and female's body contains more fat than males. This may have caused more absorption of BPA in females leading to more receptor activation **[39]**. These results were reflected on the histological picture of ovaries of both artificial saliva and citric acid groups which showed histological alteration due to hormonal changes as a result of estrogen receptor activation.

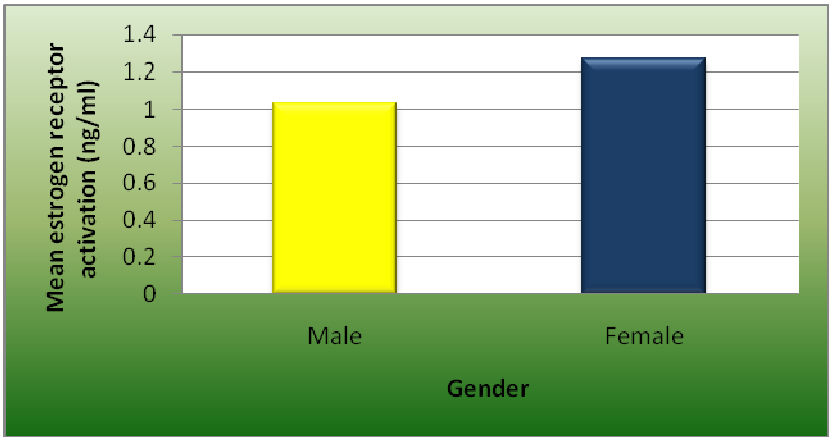

Fig. (4): Comparison between estrogen receptors activation (ng/ml) in males and females

The histological features of the tested female mice ovaries after one week administration period of artificial saliva showed different stages of the follicles and corpus luteum in the cortex. The histological findings of the present study were consistent with the results of the study made by *Adewale et al* **[40]**, who observed that females' ovaries exposed to BPA displayed all stages of follicular development. However, the previous study reported the presence of hemorrhagic follicles and a number of large antral-like follicles. Although these hemorrhagic follicles were not found in the current results, other blood vessels-related findings were identified as shown in **Fig.(5).**

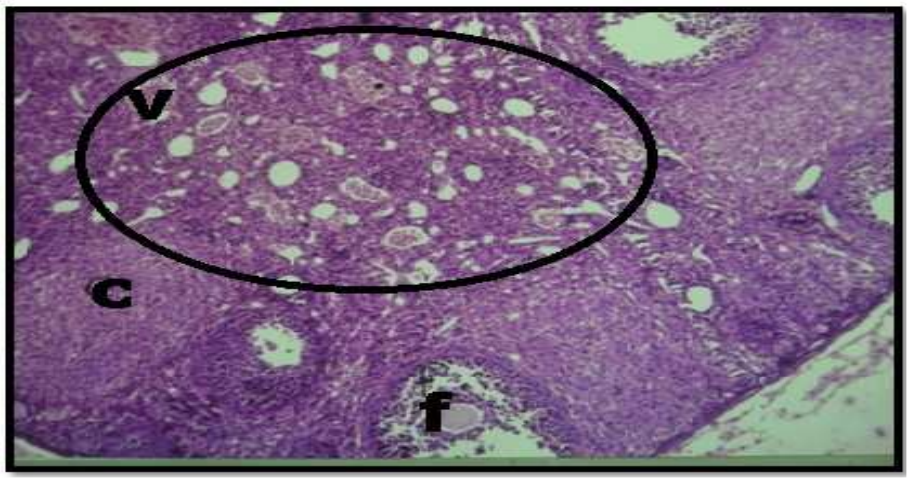

Fig. (5): Photomicrograph of an ovary tissue section of female mouse, as a representative of artificial saliva subgroup after one week showing graffian follicles (f) and corpus luteum (c) at the cortex with congested blood vessels (v) in medulla (circled area) (H&E 16 x)

The mice ovaries in the current study showed congested blood vessels of the medulla. These findings can be attributed to the fact that the estrogen has a vasodilating effect, which can result in the accumulation of blood in the veins of the organs in the pelvic area including the ovaries and the uterus **[41]**. This vasodilating effect is mediated by the estrogen – sensitive cell receptors (ERα and ERβ), which are present in the vascular endothelial and smooth muscle cells that are found in the wall of the blood vessels. Additionally, estrogen can weaken the vein walls **[42]** leading to the changes that cause varicosities that have been identified in the histological sections. Noteworthy, that the difference between the result of the current study and those reported by *Adewale et al* **[40]** may be attributed to the different ages of the mice at which the BPA was administered. In the current study, BPA was administered in pre-pubertal stage unlike the other study in which the mice were exposed in the neonatal stage. Neonatal BPA exposure is known to disrupt ovarian development **[40]**.

On the other hand, after one week administration period, female citric acid subgroup showed more pronounced changes where the ovaries showed proliferation of the interstitial stromal cells of the medulla that was not observed in the artificial saliva subgroup **(Fig.6).**

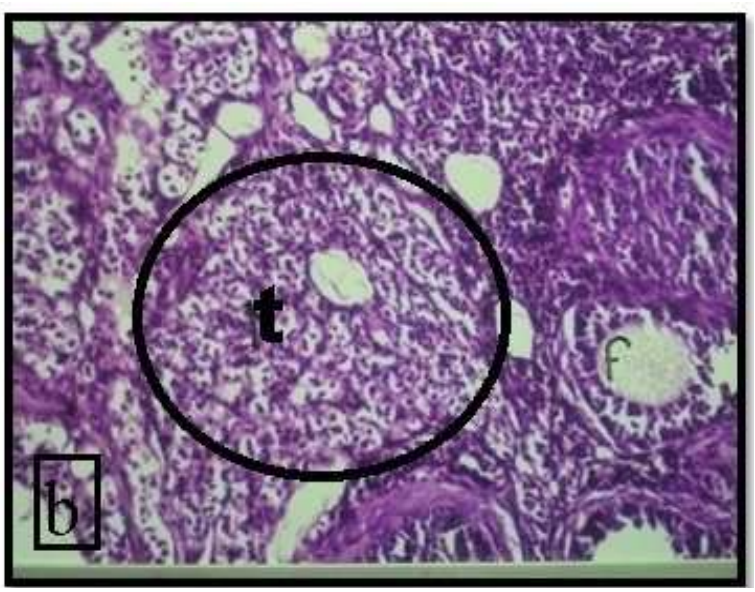

Fig (6): Photomicrograph of an ovary tissue section of female mouse, as a representative of citric acid subgroup after one week showing graffian follicles (f) and corpus luteum(c) at the cortex with proliferation (circled area) of interstitial stromal cells in the medulla (t) (H&E x 40

This can be explained by the fact that interstitial stromal cells of the ovaries contain high number of ERα receptors and BPA is known to have high affinity to bind to these receptors. These cells are known to proliferate under the effect of estrogen hormone **[43]**. These results are relevant to those described by *Park et al.* **[44]** who observed that BPA increased cell proliferation of interstitial stromal cells of the ovary through increasing the estrogen response element (ERE) activity on the DNA. This cellular proliferation decreased after two weeks to severely congested blood vessels of the ovaries while the hormonal imbalance subsided after three months to normal histological morphology.

It has been observed that, all the histopathogical changes that were observed in the ovaries of female mice of artificial saliva subgroup after one week subsided after two weeks and three months**.** This could be due to the neglected amounts of BPA in the administrated immersion media after day six. This is consistent with previous study which indicated that the amount of circulating BPA in blood is related to the intensity of the ovarian disease in women **[45]**.

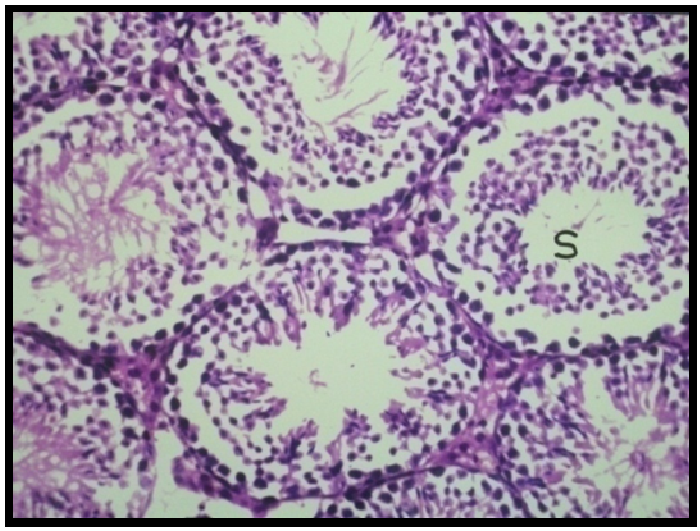

Fig. (7): Photomicrograph of testis tissue section of male mouse, as a representative of test groups (C1x artificial saliva x 2 weeks) showing normal histological structure of mature active seminiferous tubules (S) (H&E 40 x) similar findings were observed in all other variables combinations

On the other side, the histological examination of male mice testes showed no histopathological alteration. Normal histological structure of the mature active seminiferous tubules with complete spermatogenic series were observed in male mice for all materials among the two subgroups of artificial saliva and citric acid and along all the study periods (one week, two weeks, and three months) as shown in **Fig.(7).**

These results are contradictory with those reported by *Al-Hiyasat et al* **[46]** who found that leached components from resin-based dental composites significantly affected the fertility of male mice and reduced the weights of the testes and the seminal vesicles as well. This difference may be attributed to the fact that this latter study used large specimens' size compared to the animal weights. And also utilized 96% ethanol for extraction of composite and each specimen was sonicated daily for two hours prior to immersion in ethanol. All these factors are considered extreme conditions for extraction of BPA which are not representative of the less aggressive oral environment.

CONCLUSION

Based on the results obtained from the present study, the following conclusions could be drawn: (a) BPA can be effectively released from composites and sealants, (b) thermocycling and acidic pH enhance the release of BPA from resin based restorative materials , (c) the release of BPA is confined to the first six days after polymerization of the specimens and female mice were more affected than males, (d) the released BPA from the tested materials with maximum doses 417.5 micrograms had no effect on testis of male mice and (e) the estrogenic effect that might be induced from a newly placed restoration or sealant will decrease over time. However, such a conclusion cannot exclude some additive or synergistic effect with other xeroestrogens present in the oral environment.

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