UNIVERSITI PUTRA MALAYSIA

HISTOPATHOLOGICAL ANALYSIS OF BISPHENOL A (BPA) EFFECTS ON MULTIORGAN IN COLORECTAL CANCER ANIMAL MODELS

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ABSTRACT

Histopathological Analysis of Bisphenol A (BPA) Effects on Multiorgans in Colorectal Cancer Animal Model

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Introduction: Bisphenol A (BPA) is one of the most widely produced chemicals in the world, specifically used to manufacture plastics, epoxy resins, and other polymeric materials. Interestingly, BPA has been recognized as an endocrine disruptor chemical (EDC) for possessing a similar structure to diethylstilbesterol (DES), a potent oestrogen receptor (ER) agonist. Consequently, BPA can interact with ER, which is also expressed in typical colonic epithelium, predominantly by ERβ with no or limited expression of ERα. Thus, BPA is known to act as a carcinogen, increasing tumorigenesis in colorectal cancer (CRC). To date, *in vitro* migration and invasion of CRC cells triggered by BPA have been identified, but little is known about *in vivo* metastasis of cancer mentioned above to surrounding organs. **Objective:** To further explore the metastasis of CRC, this study aims to investigate the effects of BPA on histological changes in the kidney, spleen, and liver in the CRC model. **Methodology:** Twenty-four Sprague Dawley rats were separated into four groups, where A acted as a control, whereas B was administered with 25 mg/kg BPA. C was treated with 40 mg/kg 1, 2-dimethylhydrazine (DMH), while D was administered with both BPA and DMH; DMH was administered subcutaneously once per week for ten weeks to induce CRC, whereas BPA was dissolved in olive oil before orally administered for twenty weeks. Spleen, kidney, and liver were removed during dissection and fixed in formalin for histopathological examination. Morphological changes in the specimens were observed and assessed for histopathologic scoring. **Results:** No metastatic cells from the colon were observed in any of the organ samples. Pearson's Chi-Square revealed a statistically significant association between treatment groups and histopathological changes in the multiorgan observed. From the histopathological evaluation and scoring, liver, spleen, and kidneys showed significant differences from the control. In the liver and spleen, the BPA-treated group show no significant differences in microscopic changes compared to the BPA-DMH group. In the kidney, the BPA group is significantly different in histological changes compared to the BPA-DMH treated groups. **Discussion:** The absence of metastatic cells in organ samples indicates that sub-chronic oral exposure to low-dose BPA does not result in CRC progression to the multiorgan. Nonetheless, the microscopic alterations observed on the tissue samples suggests that exposure to low-dose BPA may exhibit toxic effects on the multiorgan. **Conclusion:** From the histopathological findings, **s**ub-chronic exposure of low-dose BPA can exhibit toxic effects to multiorgan by altering their typical tissue architectures but it does not trigger the progression of CRC cells to the liver, kidney, and spleen. However, the effects of BPA exposure should be further dissected to comprehend the

mechanisms underlying the toxic effects exhibited to the multiorgan and its potential to trigger the proliferation and progression of CRC cells to surrounding organs.

Keywords: Bisphenol A, Sprague Dawley, liver, spleen, kidneys, metastasis, colorectal cancer

ABSTRAK

Analisis Histopatologi Kesan Bisphenol A (BPA) pada Multiorgan dalam Model Kanser Kolorektal

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Pengenalan: Bisphenol A (BPA) merupakan salah satu bahan kimia signifikan yang banyak dihasilkan seluruh dunia dan telah digunakan sebagai salah satu bahan penting dalam menghasilkan plastik dan beberapa bahan polimer yang lain. BPA telah dikategorikan sebagai bahan kimia pengganggu endokrin kerana memiliki struktur yang sama seperti 'diethylstilbesterol' (DES) yang merupakan agonis reseptor estrogen (RE) yang kuat. Justeru, BPA mampu berinteraksi dengan RE di epitelium kolon yang didominasi oleh REβ dan sedikit atau hampir tiada REα. Oleh itu, BPA telah dianggap sebagai karsinogen yang menyebabkan tumorigenesis kanser kolorektal meningkat. Dasawarsa ini, kajian terhadap kesan BPA pada migrasi dan invasi oleh sel kanser kolorektal telah banyak dijalankan tetapi metastasis kolorektal kanser ke organ sekeliling dalam kajian in vivo masih belum diberikan perhatian. **Objektif:** Kajian ini dijalankan bertujuan untuk mengenalpasti kesan BPA pada histologi hati, buah pinggang dan limfa demi menjelajah dengan lebih mendalam metastasis kanser kolorektal. **Metodologi:** Dua puluh empat tikus 'Sprague Dawley' telah dibahagikan kepada 4 kumpulan. Kumpulan A merupakan kontrol. Manakala, kumpulan B diberikan 25mg/kg BPA. Tikus dalam Kumpulan C pula hanya diberikan 40mg/kg '1, 2-dimethylhydrazine' (DMH) untuk merangsang kanser kolorektal pada tikus. Kumpulan D pula diberikan kedua-dua BPA dan DMH. Tikus tersebut diberikan DMH secara suntikan '*subcutaneous*' sekali seminggu selama 10 minggu. BPA pula dilarutkan bersama-sama minyak zaitun sebelum diberikan secara oral kepada tikus selama 20 minggu. Hati, buah pinggang dan limfa akan diasingkan setelah diseksi dan diletakkan ke dalam formalin sebagai persediaan untuk pemeriksaan histopatologi. Perubahan morfologi pada specimen akan disiasat dan dinilai untuk skoring. **Hasil:** Tiada sel metastasis daripada kanser kolorektal pada semua organ. Analisis statistik membuktikan bahawa bahan rangsangan berkait dengan perubahan histologi pada organ. Berdasarkan analisis histopatologi dan skoring, hati, buah pinggang dan limpa menjukkan perubahan siginifikan pada morfologi berbanding kontrol. Namun, hati dan limpa Kumpulan B tidak menunjukkan perubahan histologi yang signifikan berbanding Kumpulan D. Manakala, buah pinggang Kumpulan B menunjukkan perubahan morfologi yang signifikan berbanding Kumpulan D. **Perbincangan:** Tiada sel metastasis hadir pada organ kajian membuktikan bahawa dos rendah BPA tidak merangsang tumorigenesis kanser kolorektal ke organ berdekatan. Walau bagaimanapun, perubahan mikroskopik yang terdapat pada tisu telah membawa kepada andaian bahawa dos rendah BPA memberi kesan toksik kepada organ berdekatan. **Kesimpulan:** Hasil kajian histopatologi telah mendapati bahawa tiada sel metastasis daripada kanser kolorektal hadir pada mana-mana organ pilihan kajian. Namun demikian, dos rendah BPA mampu merangsang perubahan pada tisu organ hati, buah pinggang dan limpa. Justeru, kesan terdedah kepada BPA perlu dikaji

dengan lebih lanjut untuk memahami mekanisme kesan toksik dan potensi bahan kimia tersebut dalam merangsang proliferasi sel kanser kolorektal ke organ berdekatan.

Kata kunci: Bisphenol A, Sprague Dawley, hati, buah pinggang, limpa, kanser kolorektal, metastasis

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LIST OF ABBREVIATIONS

ABS	Acrylonitrile-butyl-cyanide
AOM	Azoxymethane
AR	Androgen receptor
BPA	Bisphenol A
CDC	Centers for Disease Control and Prevention
CKD	Chronic kidney disease patients
CRC	Colorectal cancer
DES	Diethylstilbesterol
DGEBA	Diglycidyl ether of bisphenol A
DMSO	Dimethyl sulfoxide
DMH	1, 2-dimethylhydrazine
DPC	Dipropyl carbonate
E2	17-beta oestradiol
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
EDC	Endocrine disruptor compound
EFSA	European Food Safety Authority
ER	Oestrogen receptor
FCM	Food Contact Materials
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
GPER	G-protein-coupled oestrogen receptor
H&E	Haematoxylin and eosin

HCC	Hepatocellular carcinoma
HT	Hydroxytyrosol
IACUC	Institutional Animal Care and Use Committee
LOAEL	Lowest observed adverse effect level
NAFLD	Non-alcoholic fatty liver disease
NIEHS	National Institute of Environmental Health Sciences
PALS	Periarteriolar lymphoid sheath
PC	Polycarbonate
PhIP	2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine
SD	Sprague Dawley
TCSA	Toxic Substances Control Act
TDI	Tolerable Daily Intake
THR	Thyroid hormone receptor
U.S. EPA	U.S. Environmental Protection Agency

CHAPTER 1

INTRODUCTION

1.1. Background

Generally, bisphenols are a large class of chemical constituents consisting of two functional groups of hydroxyphenyl within molecules which have been extensively utilised in the manufacturing of essential products such as food storage containers, water bottles, baby food bottles, kitchen utensils, and medical consumables. Bisphenols are especially useful in the production of polycarbonate (PC) plastics and epoxy resins. Thus, trace bisphenols could be present in the environment and food if released during production or leach from products when discarded in landfills after use (Y. Q. Huang et al., 2012).

Bisphenol A (BPA) is the most extensively studied type of bisphenols and has been utilised widely in plastics, food and beverages can linings as well as thermal paper (Farris, 2014). Bisphenol A (2,2-Di (4-hydroxyphenyl) propane) was first synthesised in 1891 by Alexander P. Dianin. He succeeds in producing the constituents by discovering a condensation method in which one acetone molecule is combined with two phenol molecules in the presence of an acid catalyst (Rubin, 2011a).

According to Ambreen et al. (2019), mankind has been exposed to BPA through various routes including food and drinking water ingestion, ambient and indoor air inhalation, dust, and direct contact with BPA-containing products. Y. Q. Huang et al. (2012), claimed that BPA in the environment is man-made rather than naturally occurring. In addition, they further clarify that BPA can be released during the

manufacturing process of BPA-containing products, either through disposal of used products in landfills or through possible factory discharge.

However, BPA has been categorized as an endocrine disruptor compound (EDC) due to its estrogenic properties. The chemical possesses a similar molecule structure to diethylstilbesterol (DES), a potent oestrogen receptor (ER) agonist which can allow and block natural hormones from binding to their receptors and/or mimics hormones to disrupt the regulation of endogenous hormones (Hafezi & Abdel-Rahman, 2019). EDC can be defined as an exogenous substance that is capable of interrupting the endocrine system resulting in health-related issues in an organism (Legeay & Faure, 2017). BPA is a weak xenoestrogen compared to oestradiol in terms of binding affinity to ER; however, its ability to trigger cellular responses at lower concentrations has raised concerns (Hafezi & Abdel-Rahman, 2019).

Previous studies have demonstrated that BPA is capable of leaching from the products manufactured by using the chemical constituents as the building blocks, especially food storage containers and infants' bottles (Jenkins et al., 2012). In addition, they emphasised that oral consumption is believed to be the primary route of BPA exposure in humans. Consequently, BPA has been traced in detectable amounts either under usual or extreme condition of usage, leaching from the various daily life products containing the chemical.

Multiple diseases and health issues including diabetes, cardiovascular disease, reproductive problems as well as metabolic disease, have been reported to be associated with increased BPA concentrations in adults (Rubin, 2011). According to Hafezi & Abdel-Rahman (2019), BPA also plays a role in promoting carcinogenesis which is assumed to be due to its oestrogenic properties. BPA has been reported to induce breast and prostate cancer in animals and possibly cause these cancers in

humans (Michałowicz, 2014). In addition, Chen and Iverson (2012), clinical and experimental evidence demonstrated that oestrogens and xenoestrogens interact with specific receptors to contribute to the pathogenesis and progression of colorectal cancer (CRC).

1.2. Problem Statement

BPA has been determined to possess the ability to interact with oestrogen receptors (ER) which are also expressed in the colon, mimicking the action of endogenous oestrogen. In addition, BPA can also act as a carcinogen, promoting tumorigenesis and progression of colorectal cancer by inducing epithelial to mesenchymal transitions of cancer cells. However, the effect of BPA on *in vivo* metastasis of colorectal cancer to surrounding organs has not been fully elucidated in previous studies.

1.3. Justification

Previous findings only highlighted *in vitro* migration and invasion of colorectal cancer cells triggered by BPA. Thus, there is a need to do this study to understand the role of BPA on *in vivo* metastasis of colorectal cancer to surrounding organs.

1.4. Objectives

1.4.1. General Objectives

To further explore metastasis of colorectal cancer, this study generally aims to identify the effect of BPA on histopathology of liver, spleen and kidney in colorectal cancer model.

1.4.2. Specific Objectives

1. To determine the metastasis of colorectal cancer through observation of morphological changes in liver, kidney and spleen in colorectal cancer model.
2. To analyse semi-quantitatively histopathological changes of liver, kidney and spleen in colorectal cancer model.

1.5. Hypothesis

BPA plays a role on metastasis of colorectal cancer via histopathological changes on liver, kidney and spleen in the colorectal cancer animal model.

CHAPTER 2

LITERATURE REVIEW

2.1. Bisphenol A

2.1.1. Introduction to Bisphenol A

Bisphenol A (BPA), an organic compound that has been extensively produced worldwide, is commonly utilized as the building block in producing thermal paper, PC, and epoxy resin (Rubin, 2011). Statistically, the author mentioned that approximately 10 billion pounds of BPA is produced each year, and 100 tons of it may be discharged into the atmosphere.

According to Metz (2016), despite being discovered in the late 1800s, BPA was not commercialised until scientists successfully mixed it with plastics producing PC in the early 1900s. Due to the significant industrial production of BPA, humankind is at risk of being exposed to the chemical, directly or indirectly, resulting in detectable concentrations of the chemical approximately less than 10.6ng/ml in their body (Ohore & Songhe, 2019). The authors also highlighted that daily consumption of BPA is approximately 30.76ng/kg per body weight via various oral, dermal, and respiratory routes. Considering that European Food Safety Authority (EFSA) has revealed that the recent Tolerable Daily Intake (TDI) of BPA is only 0.06ng/kg per body weight per day, the consumption of BPA daily in humans reported by Ohore and Songhe (2019) is highly concerning.

BPA has also been widely utilised as Food Contact Materials (FCM), which referrs to any materials used to directly contact food (Mansilha et al., 2013). The authors further affirmed that the advantage of BPA as a food coating is that it ensures

a long food shelf-life. It also prevents food from being in direct contact with other chemicals, physical and microbiological components of cans. However, according to Viñas et al. (2012), bisphenol compounds can leach directly into the food from the chemical container when the packages are heated under extremely high temperatures. Consequently, more than 90% of adults in the United States acquired particular concentrations of BPA in their urinary samples (Shankar et al., 2012a). Moreover, a high amount of BPA has been determined to be presented in various environmental media, including ground and marine water, following its extensive usage in daily life (Huang et al., 2012; Ohore & Songhe, 2019).

BPA was first determined to benefit the plastic industry as early as the 1940s (Rubin, 2011). The author further emphasised that PC is frequently used in manufacturing food and beverage storage containers such as infant bottles and functions as additives in other plastics. On the other hand, the author also mentioned that epoxy resins coat the inner wall of cans and beverage containers for extended shelf life. Apart from that, the inner coat also helps to avoid direct contact of the canned food with the metallic surface (Y. Q. Huang et al., 2012). Furthermore, the authors stress that BPA possesses excellent mechanical and high thermal stability properties, which are advantageous for the mass production of daily life products such as medical equipment, reusable bottles, and food containers.

Diglycidyl ether of bisphenol A (DGEBA) is a standard commercial epoxy resin oligomer found to be utilised in the industries where almost the majority of epoxy resin production worldwide is manufactured by BPA and epichlorohydrin reaction catalysed by primary catalyst (Saba et al., 2016). Apart from low-cost properties, the authors also stress the other advantages of epoxy resins which have

been commercially utilised as coating material in the manufacturing industry, are their versatile properties and good adhesion characteristics with many substrates.

Considering the high demand for PC and epoxy resins production, BPA has been manufactured and utilised in significant volume resulting in its global market valued at over USD 10.92 billion in 2020 (*Global Bisphenol A (BPA) Market Report and Forecast 2021-2026*, n.d.). The report further clarifies that increasing demand for this chemical in the automotive industry is expected to propel the market's growth at a robust rate of 7.8% during the forecast period. The increasing passenger of vehicles also influences the surging demand for BPA in the automotive field as more production of cars' essential parts such as bumpers, headlights, and dashboards from PC resin are needed (*Global Bisphenol A (BPA) Market Report and Forecast 2021-2026*, n.d.; Saba et al., 2016).

BPA with the molecular formula of $C_{15}H_{16}O_2$ is an organic compound and derivative of diphenylmethane consisting of 2 hydroxyphenyl groups. It is a colourless solid with 228.29g/cm^3 molecular weight and is soluble in organic solvents but poorly soluble in water (National Center for Biotechnology Information, 2022). According to Michałowicz (2014), BPA has the property of a white, crystalline solid substance with a 156°C of melting point and 220°C of boiling point.

BPA is classified under phenol groups consisting of hydroxyl residue attached to the aromatic ring (Figure 1). Good reactivity characteristics of BPA can be determined through hydroxyl groups (Michałowicz, 2014). However, after investigating its biological properties, Dodds and Lawson (1936) have identified oestrogenic properties in BPA. They further clarified that the OH functional groups in the chemical structure of BPA are correlated with the oestrogenic properties of the chemical.

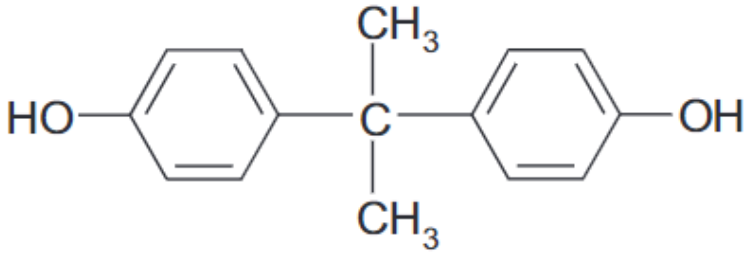


Figure 1. Chemical structure of Bisphenol A. Hydroxyl groups bind to aromatic ring presents in the chemical structure of BPA. (Adapted from: Michałowicz, 2014.)

2.1.1. Worldwide Bisphenol A Production and Consumption

Initially, BPA was only utilised for PC plastic production in the United States which later shifted to epoxy resins (Corrales et al., 2015). The authors further affirmed that the increasing demand for BPA in manufacturing products including food, and beverage containers, medical equipment, automobiles and electronics is a result of the modernisation of countries. In 2021 alone, BPA sold approximately 6,000 kilotons despite the COVID-19 pandemic occurring. The BPA market was indeed impacted in 2020 but managed but it recovered the following year.

More than 75% of the global share was dominated by PC resins for BPA market demand to produce high-quality thermoplastic for the construction industry. The increasing and strong demand for PC and epoxy resin from different industries are most likely driving the growth of the BPA market worldwide.

Increasing market of Acrylonitrile-butyl-cyanide (ABS), a high-performance thermoplastic made of BPA as one of its substances and commonly used in the building and construction industry, has led to a rise BPA demand. ABS has been substituted for glass in various applications.

Asia-Pacific nations are the leading producers and consumers of BPA, so it was anticipated that they would dominate the market. In addition, the explosive growth of PC and BPA manufacturing in China appeared to be the driving factor behind the rising demand for BPA. Zhangzhou ChimeI Chemical Co. announced in September 2021 a new PC facility that will utilise the Versalis dipropyl carbonate (DPC) technology. Furthermore, European nations have adapted the use of PC materials for greenhouse cultivation, which also has increased the demand for BPA in PC production (*Bisphenol A Market | 2022 - 27 | Industry Share, Size, Growth - Mordor Intelligence*, n.d.).

2.1.2. Toxicokinetic of Bisphenol A

BPA is directed first into the gastrointestinal tract (GIT) where it will be absorbed and metabolised in the intestine followed by the liver before reaching target organs and tissues such as the uterus and testes. The industrial chemical undergoes glucuronidation within the intestinal walls prior to being converted to inactive metabolites of BPA known as BPA-GA. The BPA-GA will then be excreted into the small intestine mucosa and colon serosa (Iwano et al., 2018).

Phase II metabolism then converts bisphenol A-glucuronide or sulphate conjugates into a highly soluble metabolite known as bisphenol A-glucuronide. BPA also has been discovered possessing the ability to be converted into various metabolites via Phase II oxidation process. However, there have been no reports of bisphenol A-glucuronide interfering with hormonal activity. Nonetheless, unconjugated BPA is able to bind to ERs upon oral administration. The “free” unconjugated and active BPA has shown that not all BPA is metabolized completely. Moreover, oral administration of BPA possesses the lowest bioavailability

characteristics compared to other routes of administrations including intraperitoneal and subcutaneous.

Lastly, BPA will be removed through urine after four to forty-three hours (Trasande et al., 2012). It has been determined that patients with damaged kidneys are unable to eliminate BPA completely, resulting in a buildup of BPA in the systemic circulation (Moreno-Gómez-toledano et al., 2021). Consequently, BPA level in plasma and tissue are significantly high in patient with renal problems. The authors have stressed the role of BPA in triggering the renal damage or disease development due to the fact that the aforementioned industrial chemical may capably bind to protein, resulting in the accumulation of a high level of albuminuria. Following that, an increase in urinary BPA was likely correlated with an increase in albuminuria which serves as an early indicator of renal damage.

2.1.3. Exposure Route of Bisphenol A in Human

BPA has been determined to be migrated into the natural environment and food chain due to human activity itself (Michałowicz, 2014). This statement is also further emphasised by Huang et al. (2012), which stressed that BPA does not occur naturally in the environment but is the result of human activity. However, the author clarified that there is evidence that BPA can be degraded over time through a rapid photooxidation process in which the byproducts are released into the atmosphere. Other than that, PC and epoxy resins products containing residual unreacted BPA may also migrate into the natural environment and food.

The manufacturing, treatment, processing, and degradation of BPA-containing products can result in the migration of BPA into the human environment

(Mercea, 2009). PCs are capable of leaching BPA via diffusion and degradation (Michałowicz, 2014). In contrast to degradation, however, the rate of BPA migration via diffusion is lower. According to Mercea (2009), a solution in a plastic water container would have a low pH as the level of BPA leached from the plastic increased. The author further discovered that the migration rate of BPA from PC water containers is higher when subjected to a higher temperature. Following that, several countries, including Canada, were among the first to prohibit BPA's utilization in infant bottles in 2010 (Rogers et al., 2013). The author further mentioned that the decision was made upon considering the detectable presence of BPA in fluids associated with pregnancy and breast milk. Furthermore, The European Commission also stopped the utilisation of BPA in infant bottles manufactured a year later, and The Food and Drug Administration (FDA) prohibits the use of BPA as a building block for infant-related products including bottles, lids or cups.

Besides that, BPA can also be migrated from the lining of cans into food stored inside (Michałowicz, 2014). Therefore, canned foods are most likely to contain BPA that has leached from lacquer coatings comprosed of epoxy resins. In addition to preventing the metal of the can from corroding, the resins functioned to prevent the food contamination during sterilization and storage by metallic substances. Michałowicz (2014), further clarified that approximately less than 23µg of BPA per tin can migrate into the food or substances packaged inside when subjected to high temperature, commonly 100°C, during the thermal pasteurization step. The statement also has been further supported by Noureddine El Moussawi et al. (2017), who determined that the BPA levels in cans subjected to high temperatures during the sterilisation process are significantly higher. Therefore, it can be concluded that high temperatures are associated with BPA leaching from coating linings.

According to Pielichowski and Michalowski (2014), BPA can also be ingested through occupational, dietary, and environmental exposures. Considering the release of BPA from various BPA-containing products used daily, it has been determined that ingestion is the primary route of chemical exposure for humans. According to Geens et al. (2014), humans are most likely to be exposed to BPA through ingestion, where it leached from BPA-containing containers or epoxy resins coating that comes into direct contact with food or water. Moreover, according to Mileva et al. (2014), extensive consumption of canned materials is strongly correlated with their economic value, resulting in their use in the vast majority of developed nations. Consequently, as mentioned above, more individuals were exposed to the chemical through ingestion.

2.2. Bisphenol A: An Endocrine Disruptor

2.2.1. Introduction to Endocrine Disruptor

According to the Endocrine Society, endocrine-disrupting chemicals (EDC) can be defined as an exogenous chemical (or a mixture) that influence normal hormone function or action through various mechanisms, including mimicking hormones, reducing hormone secretion or degradation, transforming hormone receptors, or blocking the normal binding of the receptors. BPA is classified as environmental EDC as it was released anthropogenically or during production. According to Kumar et al. (2020), EDC can disrupt the homeostasis of hormones, resulting in reproductive problems, development of neuron and growth cancers, and immune-related issues.

The European Commission has outlined several characteristics of chemicals to be defined as EDC, which can exert endocrine activity, disrupt regular endocrine activity and acquire stimulus-response association between substance and endocrine activity. EFSA proposed that most EDCs are synthesised to interfere with the normal binding of endocrine receptors or gene expression.

EDCs may be introduced to mankind through inhalation, ingestion and dermal contact (Lauretta et al., 2019). The author further emphasised that most EDCs formed a mass in adipose tissue due to their lipophilic property, which allows for a longer half-life.

2.2.2. Mechanism of Bisphenol A Action on Endocrine Receptor

According to Lauretta et al. (2019), BPA interferes with the normal function of the endocrine system through epigenetic changes, including methylation and/or acetylation of DNA and histone modification (Figure 2). According to Hafezi and Abdel-Rahman (2019), BPA is classified as an EDC due to its exogenous oestrogen properties that disrupt and block normal endogenous hormones from binding to their respective receptors by mimicking the natural hormones.

BPA can bind to ER to exert oestrogenic effects in the body (Rochester, 2013). Despite its low affinity for the receptors, the author clarified that the oestrogenic effects of BPA are comparable to those of endogenous 17-beta oestradiol (E2) upon binding. BPA is therefore able to inhibit the normal hormonal pathway by preventing the normal binding of endogenous E2 to ER and endogenous androgen to their specific receptors. The author also stated that BPA could bind to thyroid receptors and prevent the normal binding of endogenous thyroid hormones.

The reproductive system is easily affected by BPA due to its ability to disrupt the normal function of reproductive hormones (Frenzilli et al., 2021). However, according to Jenkins et al. (2012), BPA exerts a different affinity for two types of ERs found in the body, which are ER-beta (ERβ) and ER-alpha (ERα). The author further clarifies that BPA possesses a higher affinity towards ER-beta (ERβ) compared to ER-alpha (ERα).

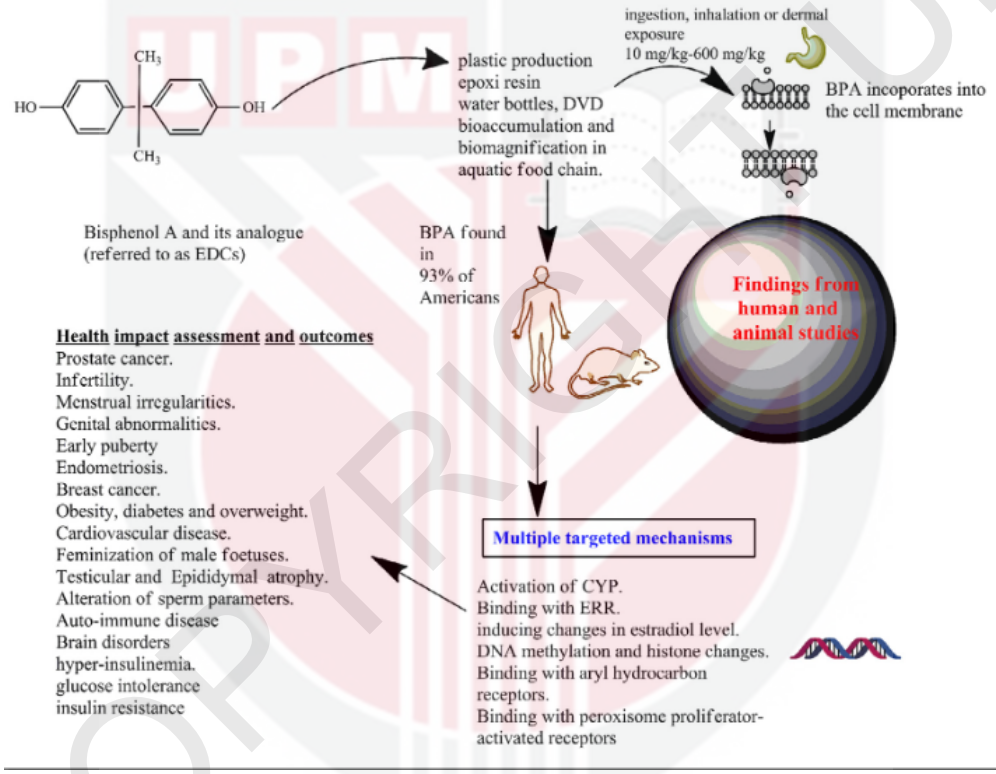


Figure 2. Endocrine activity mechanism of BPA exposure. Figure illustrates the mechanism of endocrine activity exerted by BPA upon exposure. (Adapted from *Ohore & Songhe, 2019*).

2.3. Detrimental Effect of Bisphenol A Exposure towards Human

2.3.1. Bisphenol A Toxicity

BPA has been determined to possess moderate acute toxicity properties to vertebrate organisms (Michałowicz, 2014). Researchers have determined that the lowest observed adverse effect level (LOAEL) of BPA is 50mg/kg body weight/day after conducting a carcinogenesis study on adult rodents by administering high doses of BPA daily for two years.

The U.S. Environmental Protection Agency (U.S. EPA) has addressed the associations between BPA and adverse effects on reproductive, developmental, and systemic systems based on numerous animal model studies and evidence demonstrating BPA's interaction with oestrogen receptors (ER). Therefore, BPA has been placed on the Concern List under the Toxic Substances Control Act (TCSA), which mandates that the chemical be regularly monitored for contamination in landfills, plants, and water in collaboration with the FDA, Centers for Disease Control and Prevention (CDC), and National Institute of Environmental Health Sciences (NIEHS). Other than that, the National Center for Toxicological Research, a division of the FDA, has established rules and measures to reduce human exposure to BPA through the food chain by encouraging companies in the food industry to manufacture products, such as baby bottles, using alternative materials.

Diabetes, cardiovascular issues, and liver enzyme level problems have all been linked to BPA exposure in humans (Rubin, 2011). The effects of BPA on human systems are summarised in Table 1 below.

Table 1. Health-related issues associated with BPA exposure

Categories		Study type	Results	Reference
Reproductive system	Fertility	Prospective cohort (Women with IVF treatment, n = 84)	Lacking ovarian response correlated with high level of BPA	(Mok-Lin et al., 2010)
		Case-control (Infertile women and fertile controls, n = 61)	Women with infertility issues is detected to have higher serum BPA concentration compared to controls	(Caserta et al., 2013)
	Endometrial disorders	*In vitro* (Adult female mice)	Low-dose of BPA level exposure during pubertal period associated with poor uterine function in adult phase.	(Q. Li et al., 2016)
	Sperm quality	Occupational cohort (Men workers in BPA and epoxy resins plants and unexposed controls, n = 218)	Poor sperm quality is associated with high level BPA concentration in urine	(D. K. Li et al., 2011)
		Cross sectional (Male, n =190)	High BPA concentration detected associated with poor sperm quality characteristics includes, low	(Meeker et al., 2010)

			sperm count, abnormal sperm morphology, sperm motility and DNA destruction	
Metabolic disease	Obesity	*In vivo* (mice)	Adult phase mice have significantly high body weight and adipocytes level.	(Bodin et al., 2013)
		Cross sectional (children, n = 1326)	High BPA level detected in girls with increased body weight but no similar results in boys	(D. K. Li et al., 2013)
	Diabetes	*In vivo* (pregnant mice)	Offsprings acquired low glucose tolerance and synthesis of insulin	(Liu et al., 2013)
		Cross sectional (adults, n = 1210)	High BPA level detected in the urine is correlated with high incidences of type II diabetes	(Kim & Park, 2013)
	Heart diseases	Cross sectional (Adults, n = 745)	High BPA concentration level has been determined to be correlated with peripheral arterial disease progression	(Shankar et al., 2012b)
		Cross sectional (Adults, n = 521)	High BPA level concentration in urine is determined to be correlated with hypertension	(Bae et al., 2012)
Endocrine disruption	-	*In vitro* and *in vivo* (zebrafish)	Abnormal thyrocytes	(Gentilcore et al., 2013)

2.3.2. Bisphenol A Carcinogenicity

Tumorigenesis occurs via epigenetic modifications, specifically deletion, upon exposure to BPA during the perinatal period (Birnbaum & Fenton, 2003). Investigation on Sprague-Dawley rats demonstrated that low-doses of BPA initiated tumour development and disrupted mammary gland growth (Soto et al., 2013).

According to (Hafezi & Abdel-Rahman, 2019), BPA has been determined as a carcinogen due to its ability to activate cellular responses resulting in tumorigenesis. Moreover, the author clarified that BPA had been discovered to interfere with cell proliferation, apoptosis, and migration via disrupting cell signalling pathways. The organs summarised in Table 2 below are those whose exposure to BPA leads to the development of tumours.

Table 2. Cancers associated with Bisphenol A exposure

Cancers	Study	Result	Reference
Breast	*In vivo* (rats)	Progression in mammary gland development with significantly high incidences of breast tumours	(Soto et al., 2013)
	In vitro (MCF-7 human breast cancer cells)	Breast cancer cells proliferate upon being exposed to BPA via PTTG1-dependent cell cycle pathway	(Deng et al., 2021)
	Case-control (Women, n = 52)	BPA level detected in the urine samples directly associated with BPA level measured in breast adipose tissue	(Keshavarz-Maleki et al., 2021)
Ovary	*In vitro* (OVCAR-3 human ovarian cancer cells)	Ovarian cancer cells proliferate after being exposed to low concentrations of BPA	(Sang et al., 2021.)
Prostate	*In vivo* (rats)	Positive association between BPA exposure and prostate cells proliferation through cell apoptosis inhibition	(D. Huang et al., 2017)
	In vitro (Human prostate cancer cell LNCaP)	Exposure to high concentration of BPA is assumed to trigger progression of prostate cancer via AR dependent prostate cell growth inhibition	(Bilancio et al., 2017)

2.4. Colorectal Cancer

2.4.1. Colorectal Cancer: An Overview

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, ranking third behind breast and lung cancer (Figure 3A). Besides, CRC ranked fourth among cancers that cause death (Figure 3B). Statistically, between one and two million recent cases are reported annually, with men constituting the majority of incident cases (Siegel et al., 2021). Globally reported cases of CRC are expected to exceed three million by 2040, based on a various factor, including age, population, and human growth (Xi & Xu, 2021). Future cases of colorectal cancer (CRC) are anticipated to increase due to environmental factors such as modern lifestyle and diet (Kato et al., 2014; Keum & Giovannucci, 2019).

CRC is defined as abnormal mass growth in the colon or rectum region, which is the distal segment of the large intestine in the digestive system. As the large intestine is sometimes referred to as the large bowel, CRC is also commonly known as colon cancer. The large intestine plays an essential role in absorbing water and electrolytes from food and eliminating waste. In general, there are three subtypes of CRC: rectum, ascending, and descending colon, as depicted in Figure 4 (Keum & Giovannucci, 2019; F. Y. Li & Lai, 2009). Fleming et al. (2012) stated that most CRC cases are adenocarcinomas, defined as malignant neoplasms that develop from colon and rectum glandular epithelial cells.

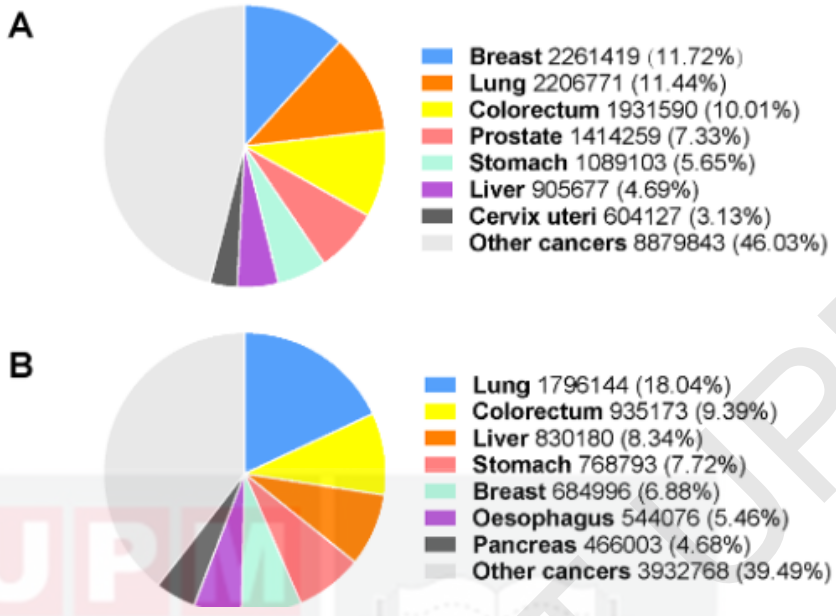


Figure 3. Estimates and proportions of recent cancer cases and deaths due to major cancers in the world in 2020. (A) Approximate number of recent cases of cancers from around the world in 2020. (B) Approximate number of deaths due to cancers from around the world in 2020. (Adapted from: Xi and Xu, 2021).

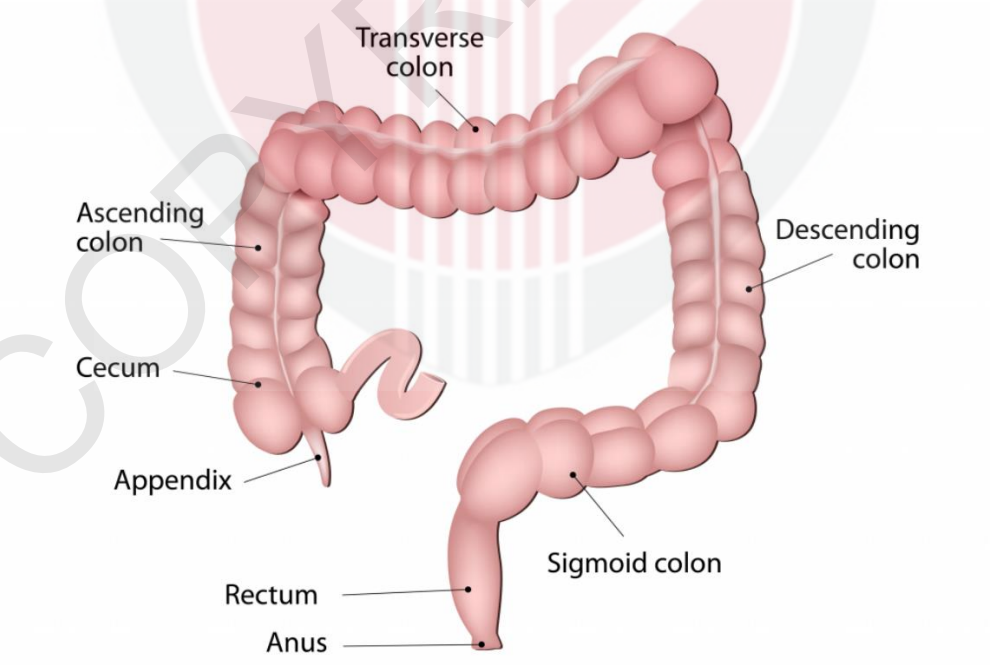


Figure 4. Large intestine, the distal part of the gastrointestinal tract. The large intestines organ extends from cecum to the rectum located at the distal colon. (Adapted from: The colon, 2022).

2.4.2. Colorectal Cancer and Bisphenol A

In addition to altering the part of the nervous system connected to the gastrointestinal tract, BPA has been found to disrupt the normal function of the intestinal barrier by inducing inflammation (Gonkowski, 2020). The author added that researchers had demonstrated the role of BPA in the initiation of cancer in the digestive tract. Because ingestion is the primary route of BPA exposure for humans, the author emphasised that digestive organs, including the stomach and intestine, are highly susceptible to this chemical.

According to Z. J. Chen et al. (2015), numerous cancer lines contain both ERα and Erβ, G-protein-coupled oestrogen receptors (GPER). Given that BPA can act on the receptors found in the colon, the authors' study demonstrates that BPA indeed plays a role in initiating and promoting colorectal carcinogenesis.

2.4.3. Metastasis of Colorectal Cancer and Bisphenol A

Jun et al. (2021) have shown that BPA influences colon cancer cell growth and migration via ERK signalling pathways. The study also has successfully determined that administration of BPA dramatically increases tumour mass growth in colorectal cancer animal models.

Moreover, an investigation performed by Z. J. Chen et al. (2015) has also demonstrated BPA triggers the CRC's metastasis through epithelial-mesenchymal transition (EMT) of cancer cells. According to Sheehan et al. (2007), EMT is the initial phase of metastasis in which cancer cells invade neighbouring cells and tissue. Therefore, the study demonstrates that BPA influences the metastasis and oncogenesis of CRC.

Given its anatomical sites and portal circulation, CRC usually metastasizes to the liver (Sheth & Clary, 2005). Less than 20% of CRC patients presented with hepatic metastasis at their initial medical consultation, while 25% of patients presented with metastasis following resection of the primary tumour (Adloff et al., 2017). The authors further justify that roughly 70% of patients suffering from colorectal cancer will eventually develop hepatic metastasis.

On the other hand, metastasis to the kidney from colorectal has been determined to be extremely rare and sporadic; even if it occurs, it manifests as concomitant carcinoma (Aksu et al., 2003). Most patients with CRC metastasis to the kidney exhibited no symptoms but were detected through imaging monitoring or elevated CEA levels (Dulskas et al., 2015). Similarly, CRC metastasis to the spleen is clinically uncommon but can occur due to disseminated disease (Abdou et al., 2016).

2.5. Animal Model

2.5.1. Colorectal Cancer Animal Model

Laboratory rodents are frequently utilised as model organisms in the experimental investigation because they are simple and inexpensive to maintain, have well-studied physiology and genetics, and are similar to humans (Nascimento-Gonçalves et al., 2021). The authors further highlighted those rodents can aid in a comprehensive understanding of disease pathogenesis and the development of clinically applicable therapeutic strategies.

According to Johnson and Fleet (2012), rat and mouse intestines are more structurally and functionally similar to human intestines. Due to their resemblance to humans in many aspects, including physiological and tumour initiation, rodents are

an ideal organism model for studying the development of colorectal cancer (CRC), despite the fact that none of the available animals precisely replicates every human disease.

2.5.2. Sprague Dawley

The Sprague Dawley (SD) rat, an outbreed albino rat, has been mainly used in biomedical, medical studies and investigations. Over the years, the SD rat has been favoured in numerous scientific studies due to its docile nature and manageability. This SD rat was first bred in Madison, Wisconsin, by SD farms, now known as Sprague Dawley Animal Company.

Rats have an average litter size of 10.5, with females weighing between 250 and 300g and males weighing between 450 and 520g. The average lifespan of a rat is less than three and a half years. The most distinguishing characteristic between SD and Wistar rats is their long tails concerning their bodies (*Sprague Dawley*, n.d.).

2.5.3. Chemically Induced Colorectal Cancer Models

Chemical agents alone or in combination can essentially induce CRC in rodents via administration (Tanaka, 2009). Direct and indirect agents are the two types of chemical carcinogens utilised to induce tumour development (Machado et al., 2016; Tanaka, 2009). The authors stated that direct carcinogenic agents are chemicals capable of inducing cancer without a metabolism. In contrast, indirect carcinogens are agents that undergo a biotransformation process to convert their inactive form to their active form before administration to exert their carcinogenic properties. The liver is specifically responsible for the conversion process.

In the early 1900s, it was discovered that dibenzanthracene or methylcholanthrene could be used to induce intestinal tumours in mice, resulting in the development of adenocarcinoma in the small intestine (Kobaek-Larsen et al., 2000). The authors further explained that years later, numerous studies have been conducted to determine which chemical agents are most effective in causing and promoting the development of colorectal carcinoma.

The chemicals that act as carcinogens to cause colorectal cancer can be classified as either direct or indirect-acting agents. These agents include N-methyl-N-nitrosourea (MNU) and N-methyl-N-nitrosoguanidine (MNNG), 1,2-dimethylhydrazine (DMH), azoxymethane (AOM), and 2-amino-1-methyl-6-phenylimidazo (4,5-b) (PhIP). In contrast to intraperitoneal administration, subcutaneous administration of DMH has been determined to be the most effective method of tumour induction (Venkatachalam et al., 2020).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1. Materials and Instruments

Table 3. List of materials and instruments used in the study

No.	Materials and Instruments	Country
1.	Bisphenol A (Tokyo Chemical Industry)	Tokyo, Japan
2.	1,2-dimethylhydrazine (DMH)	-
3.	Terumo Syringe	Japan
4.	Oral gavage needles	-
5.	Leica TP1020 Automatic Benchtop Tissue Processor, Semi-Enclosed	Germany
6.	Leica RM2255 Fully Automated Rotary Microtome	Germany
7.	Feather Microtome Blade High Profile	Japan
8.	Microscope Frosted Glass Slide	China
9.	Mounting Bath Leica HI1220	Germany
10.	Cold Plat Leica HI1130	Germany
11.	Oven Memmert ULM400	Germany
12.	Leica ST5010 Autostainer XL	Germany
13.	Leica EG1160 Tissue Embedding Station	Germany
14.	As One Coverslip glass (24 X 32mm and 18 X 18mm)	Japan
15.	Sigma-Aldrich DPX Mountant for microscopy	Germany
16.	Fume hood	-
17.	Olympus BX51 Fluorescence Microscope	Japan

18. Toup Camera and Toup View Software by Touptek China Photonics

3.2. Methods

3.2.1. Preparation of Bisphenol A and 1,2-dimethylhydrazine (DMH)

Bisphenol A (BPA) and 1,2-dimethylhydrazine (DMH) have been provided by Anatomy and Histology Laboratory 1 of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. BPA obtained in solid form, whereas DMH was in liquid form. Olive oil was used to dissolve BPA prior to administration through the oral route. On the other hand, DMH will be administered through a subcutaneous route in the groin region. The volume of BPA administered to the animals is 25mg/kg, whereas 40mg/kg per body weight for DMH.

3.2.2. Sprague Dawley and Treatment Groups

Twenty-two Sprague Dawley (SD) rats, aged four weeks and weighing 150 to 200g, were used in this study. They were housed in a facility with controlled temperature and humidity maintained on a 12 hours light-dark cycle. All animals were fed with a regular chow diet. The animals were allowed to undergo acclimatisation for one week prior to the experimenting's procedures.

The experiments conducted complied with the OECD guidelines for testing chemicals. The protocol has been proposed and approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia. SD rats were randomly divided into four groups with six animals per group or cage. Four groups were labelled as A, B, C and D where A acted as the control. B, on the other hand, was administered with BPA. Group C was administered with only DMH, and D was administered with

both BPA and DMH. The animals were sacrificed after 20 weeks of experimental phase. BPA was administered orally throughout the 20 experimental weeks. On the other hand, DMH was administered once per week for only the first ten experimental weeks.

3.2.3. Experimental Design

Table 4. Experimental design of the study

Phase	Normal chow diet																				
	Acclimatisation	CRC induction with DMH										Observation on tumour growth									Cull
		BPA Treatment																			
Animal's age (week)	4*	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Months	February				March				April			May						June			July
Experimental week	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

*Rats weighed 150 to 200g upon arrival.

3.2.4. Histopathology Examination Routine

3.2.4.1. Fixation and Grossing

Upon sacrifice, spleen, liver, and kidneys were extracted from the animals and fixed in 10% neutral buffered formalin for histopathology examination routine. Fixation is a vital step after dissection to ensure the antigenicity of the tissues is maintained. The shape of the tissues is preserved by preventing autolysis from occurring. Various types of fixatives can be used for fixation, which can be classified into five groups based on their mechanism of action: aldehydes, alcohols, oxidizing agents, mercury, and picrates. The grossing step was carried out after the fixation to obtain the areas of interest in the organs. Small pieces of liver and spleen were obtained whereas for kidneys, they were cut into half to obtain the inner surface of the organ.

3.2.4.2. Processing

The preserved tissues were processed overnight using an automatic tissue processor (Leica TP1020 Automatic Benchtop Tissue Processor). During tissue processing, the tissues were subjected to various reagents, including formalin, increasing concentration of alcohols, xylene, and paraffin wax. The purpose of tissue processing is to use different reagents to ensure the tissues were subjected to fixation, dehydration, clearing, and impregnation.

The tissue processing step in the histopathology examination routine ensures the tissues are ready for sectioning into microscopically thin ribbon sections. Thus, the process involved is via fixing the tissue into paraffin. However, wet fixed tissues are unable to be directly infiltrated with paraffin. Therefore, increasing concentrations of alcohols were used for dehydration, where water was removed from the tissues. Then,

tissues were subjected to xylene reagent three times to remove dehydrant. Lastly, the tissues were infiltrated with paraffin wax, an embedding agent.

3.2.4.3. Embedding

Embedding, also known as tissue blocking, is where the processed tissues are blocked or enclosed into an embedding medium using a mould. The embedding step occurred right after the tissue was removed from the automatic tissue processing machine. Embedding is another crucial step in preparing the tissue for the sectioning step. A supporting medium was required because the tissues had a fragile characteristic. Several embedding agents, including paraffin wax, celloidin, and gelatine, can be used. However, this study used paraffin wax as the embedding substance.

During embedding, warm paraffin wax was filled into the mould of appropriate size. The mould selected is ensured to have at least 2mm surrounding margin allowing sufficient space for the tissue to be enclosed by wax. Then, the tissue was placed according to the side to be sectioned at the centre of the mould containing the melted paraffin wax using forceps. Slight pressure is applied to the tissue to ensure even embedding.

Then, the mould was transferred to the cold plate until the paraffin was converted into semi-solid. The tissues were oriented and firmed into the wax using forceps to ensure the correct orientation was obtained, and the tissue surface to be sectioned was maintained in a flat condition. Kidneys were handled carefully when embedded compared to the spleen and liver as the inner surface needs to be ensured to face down against the mould as the part mentioned above is the interested area to be

observed. As for the liver and spleen, both organs consisted of homogenous cells on all surfaces, making embedding easier as any surface could be put against the mould.

The embedding cassette was labelled accordingly and correctly before being inserted into the mould. Then, more paraffin was added into the mould until it filled both cassette and mould. Then, the block is transferred back to the cold plate. The mould is left until the wax is completely frozen or becomes solid. Once frozen, the blocks were removed from the mould.

3.2.4.4. Trimming and Sectioning

Trimming and sectioning are where the tissue blocks are sliced into microscopically thin slices of material known as ribbon of sections, approximately 5µm, using a microtome. The extremely thin sections are very crucial to obtain in order for them to be observed with a light-optical microscope.

Firstly, the tissue block was trimmed into 14µm sections until all the surfaces of the tissues were exposed. The cutting and blade angle were adjusted when the cutting angles were uneven by turning the setscrew on the microtome. The tissue was first subjected to trim by setting the cutting thickness on the control panel to 15µm. Once the tissue surface was exposed, the tissue block was sectioned into 5µm ribbon sections.

The well ribbon sections were then put into the floatation bath. Well ribbon sections were selected and placed on a glass slide through the 'fishing' technique. The glass slides were cleaned first by wiping them to avoid any dust. Lastly, the glass slides were labelled accordingly by using 2B pencils. The slides were left overnight before staining.

3.2.4.5. Haematoxylin and Eosin staining

Upon sectioning, slides were placed in the oven with a temperature of 56°C for 20 minutes to melt the paraffin wax surrounding the samples. Then, the slides will be arranged into the staining rack before placing them into the automated staining machine. Haematoxylin and eosin (H&E) staining was performed to dye the slides red or pink and purplish-blue.

There were various reagents and chemicals used throughout the staining process. Firstly, the slides were placed inside the xylene baskets twice, for approximately 6 minutes, to deparaffinized the wax. The remaining wax was removed entirely through this deparaffinization step because paraffin wax has the characteristic of hydrophobics. Then, the slides were subjected to decreasing concentrations of alcohols for rehydration to remove the xylene for approximately 8 minutes in total. The cells and tissue elements on the slides were then hydrated and ready for penetration by aqueous reagents.

Next, the slides were washed with water for 5 minutes before being stained with haematoxylin dye for approximately 15 minutes. Then, the slides were rewashed with water. The slides were then dipped into weak ammonia 15 times before being washed with deionised water. Weak ammonia functions as a 'bluing' agent to neutralise the tissue before dipping it into acid alcohol. Acid alcohol was the next step, where slides were dipped five times for differentiation. In other words, a differentiation step is required to remove nonspecific background staining. After washing, slides were stained with eosin for 10 minutes before being subjected to an increasing concentration of alcohol for dehydration. Lastly, the clearing step then took place by dipping the slides into xylene baskets for three times.

Optimisation was done by using at least two slides of each sample before running the actual slides in the staining machine to ensure both staining reagents dyed the samples nicely. H&E staining is the most common system performed in histology due to its ability to demonstrate and allow observations of various cellular properties, including cytoplasm, nucleus, and extracellular matrix.

Eosin has acidic properties, which can stain eosinophilic structures in red or pink. Therefore, cytoplasm, including collagen, elastic and muscle fibers, as well as red blood cells, were stained pink by H&E staining. Eosin was used as the counterstain to differentiate between cytoplasm and nucleus of cells. On the other hand, haematoxylin is essential to stain basophilic structures, including the nucleus, in purplish-blue colour. Haematoxylin dyes can clearly demonstrate the details of the nucleus in the cells, including the heterochromatin and nucleoli.

3.2.4.6. Mounting

Mounting is a step where the glass slides are covered with a coverslip, sizing 24 x 24mm and 24 x 50mm, depending on the sample size on the glass slide. Mounting is a vital step in histology to physically preserve the specimen during storage, besides enhancing the quality of imaging during microscopic examination. A mounting medium is utilised to have the glass slides covered with a coverslip. The mounting medium utilised in this step was DPX which is made from a mixture of Distyrene, plasticizer, and xylene. DPX is the most commonly used mounting medium in most histology works due to its ability to preserve stain and dry faster than another type of mounting agent.

Firstly, a drop of DPX was added into the centre of the coverslip in 24 x 24mm size. Then, put the glass slides down on the coverslip and let the surface tension pull the coverslip. Gentle pressure was put on the coverslip by pressing it lightly to avoid air bubbles. Two drops of DPX were put if using a 24 x 50mm coverslip.

It is vital to utilise sufficient DPX because too little mounting medium results in air bubbles formation. Pressing down the coverslip too hard must be avoided to prevent the three-dimension structures of the sample from being distorted. If too many air bubbles form, avoid wasting time removing them but instead dip the slides back into the xylene to separate the coverslip. Then, remount the samples again.

3.2.4.7. Microscopic Examination

The mounted specimen was observed for any abnormalities and pathological changes under light microscopy. The magnifications utilised for specimen observations were 40X and100X for all organs.

3.2.5. Qualitative and Semi-Quantitative of Liver, Spleen and Kidney Pathological Abnormalities

Two methods used in analysing the mounted specimen were qualitative and semi-quantitative. Firstly, the specimens were qualitatively analysed by observing any pathological changes showing evidence of metastasis into the organs. Then, for a semi-quantitative comparison of the structural changes, the abnormalities in the tissue specimens were graded from 0, which indicates normal, to 4, indicating severe pathological changes (Ibrahim et al., 2018). Table 5 summarizes the microscopic features observed for each organ.

Table 5. Histopathological changes features of the liver, kidney and spleen

Organ	Histopathologic Feature
Liver	Steatosis
	Infiltration of inflammatory cells
	Hepatocyte degeneration
	Nuclear degenerative changes (pyknotic)
	Sinusoids dilation
	Congested blood vessels
	Dilated central vein
Kidney	Distorted glomeruli
	Dilated tubules
	Infiltration of inflammatory cells
	Tubular congestion
Spleen	Periarteriolar lymphoid sheath (PALS) numbers, size and cellularity

3.2.6. Statistical Analysis

Pearson's chi-square test was used for semi-quantitative variables analysis and applied to compare the histopathological changes between the four treatment or independent groups. Then, data of three independent animals used per group was presented in means ± S.E.M. One-Way Analysis of Variance (ANOVA) followed by post-hoc comparison using Tukey's Honest Significant Difference test. Statistical significance was defined as p-value less than 0.05 ($p < 0.05$). Statistical analysis were performed using SPSS version 26.0 (IBM).

CHAPTER 4

RESULTS

4.1. Liver

4.1.1. Metastasis

No sign of metastasis has been observed upon microscopic examination in the liver of all treatment groups.

4.1.1. Histopathological Changes and Abnormalities

The treatment effects of BPA on the animals for any abnormalities on histopathology were performed. Data were analysed by using Pearson's Chi-Square test. The statistical test has revealed a statistically significant association between treatment groups and histopathological changes in the organ observed where majority of the treatment groups demonstrated mild to moderate histopathological abnormalities in liver, as shown in Table 5 ($x(9) = 33.6, p < 0.05$).

The data was then presented as mean ± S.E.M. representing six independent animals utilised in each group in the experiment, as shown in Figure 6. The one-way ANOVA analysis of the liver's histopathological changes or abnormalities determined significant differences between groups ($F(3,20) = 40.61, p < 0.05$). A Tukey post hoc test revealed that there is statistically significant different in histopathological changes in BPA, DMH, and BPA-DMH groups compared to control ($p < 0.05$). The histopathological changes in the control group shows normal hepatic architectures (83.3%), as illustrated in Table 5.

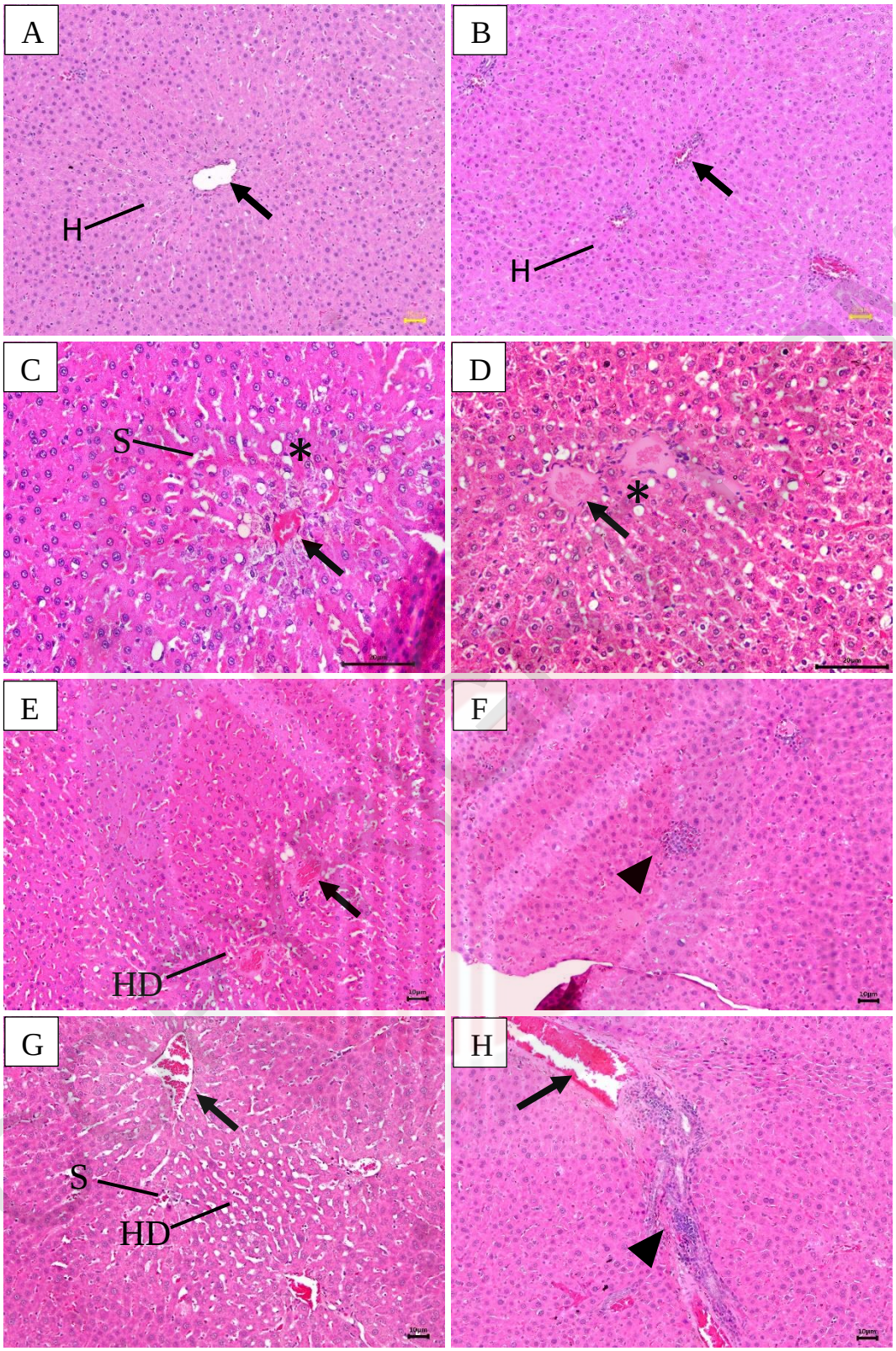


Figure 5. Photomicrograph of liver sections of control, BPA, DMH and BPA-DMH treated groups. (A-B): Sections of a control liver showing normal architecture of liver with normal central vein (arrow) and hepatocytes (H). (C-D): Sections of BPA treated liver showing dilated and congested central vein (arrow), steatosis (asterisk), dilated sinusoids (S). (E-F): Sections of DMH treated liver showing hepatocyte degeneration (HD), congested central vein (arrow) and focal inflammatory cells infiltration (arrowhead). (G-H): sections of BPA-DMH treated liver showing

hepatocyte degeneration (HD), dilated and congested central vein and portal vein (arrow), congested sinusoids (S) and inflammatory cells infiltration (arrowhead). (Magnification 100x)

Table 6. Treatment groups vs histopathological scoring (crosstabs)

Group		Histopathological Scoring					
		0	**1**	**1.5**	**2**	**2.5**	**Total**
Control	Count	5 (83.3%)	1 (16.7%)	0	0	0	6 (100%)
BPA	Count	0	0	2 (33.3%)	4 (66.7%)	0	6 (100%)
DMH	Count	0	4 (66.7%)	2 (33.3%)	0	0	6 (100%)
BPA-DMH	Count	0	0	2 (33.3%)	4 (66.7%)	0	6 (100%)
Total	Count	5 (20.8%)	5 (20.7%)	6 (25.0%)	6 (33.3%)	0	24 (100%)

Apart from that, DMH group is statistically significant different in histopathological changes as compared to BPA and BPA-DMH ($p < 0.05$) where the majority of animals in the group with colorectal cancer (1.17 ± 0.26) appeared with mild hepatocyte degeneration, congested central vein, and inflammatory cells infiltration (66.7%) in some areas of hepatic architecture as illustrated in Figure 5. On the other hand, most animals in BPA-DMH treated group (1.83 ± 0.26) appeared with mild to moderate hepatocyte degeneration, steatosis, pyknotic nucleus, inflammatory cells infiltration, dilated and congested central, portal vein and sinusoids (66.7%) as shown in Table 5. BPA group (1.83 ± 0.26) mainly exhibits moderate histopathological scoring, which occurs in moderate steatosis (66.7%). In some cases, dilated and congested sinusoids and congested central vein have also been observed,

as detailed in Figure 5. Last but not least, there was no statistically significant different in histopathological changes in BPA as compared to BPA-DMH ($p > 0.05$).

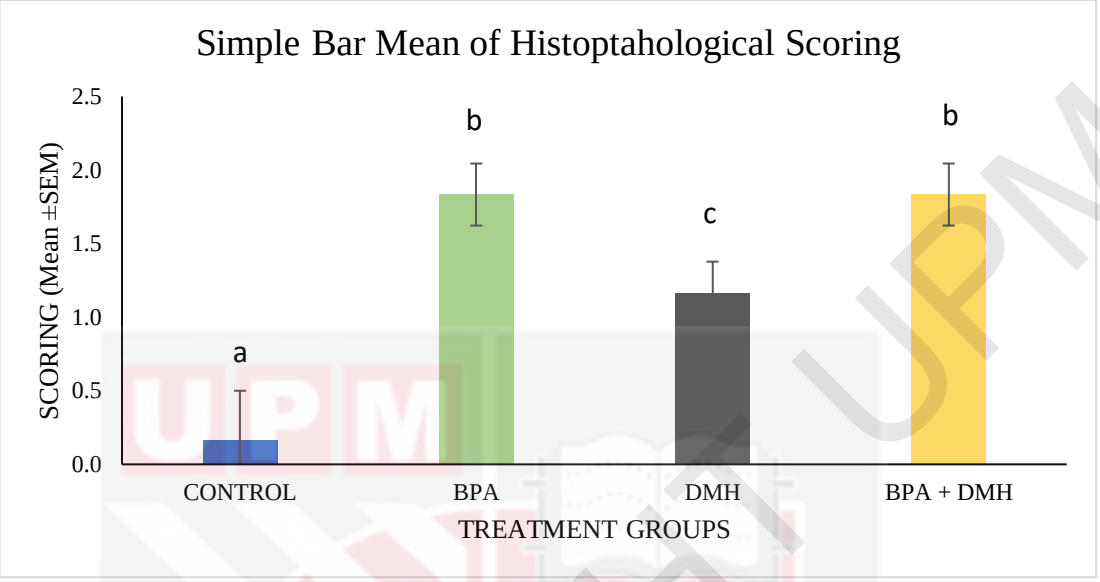


Figure 6. The bar graph shows the scoring of pathological changes in the liver. The different treatments given for different groups showed different pathological changes scoring compared to the control. Results were expressed in mean ± S.E.M of three independent animals used per group in the study. Different alphabets represent $p \leq 0.05$ significantly different from the BPA and BPA-DMH group.

4.2. Kidney

4.2.1. Metastasis

No pathological evidence demonstrating occurrence of metastasis from colorectal to kidney has been observed.

4.1.2. Histopathological Changes and Abnormalities

The treatment effects of BPA on the animals for any abnormalities on histopathology was performed. Data were analysed by using Pearson's Chi-Square test. The statistical test has revealed that there is statistically significant association between treatment groups and histopathological changes in the organ observed where

majority of the treatment groups demonstrated mild histopathological abnormalities in renal ($x(12) = 39.03, p < 0.05$) as indicated in Table 6.

The data was then also presented as mean ± S.E.M. representing six independent animals utilised in each experiment group, as displayed in Figure 8. The one-way ANOVA analysis of the histopathological changes or abnormalities in the kidney determined significant differences between groups ($F(3,20) = 63.08, p < 0.05$). A Tukey post hoc test revealed a statistically significant difference in histopathological changes in BPA, DMH and BPA-DMH groups compared to control ($p < 0.05$). Table 6 indicates that the kidney histopathology shows typical architecture of renal cortex and glomerular tufts in the control group (83.3%), as illustrated in Figure 7.

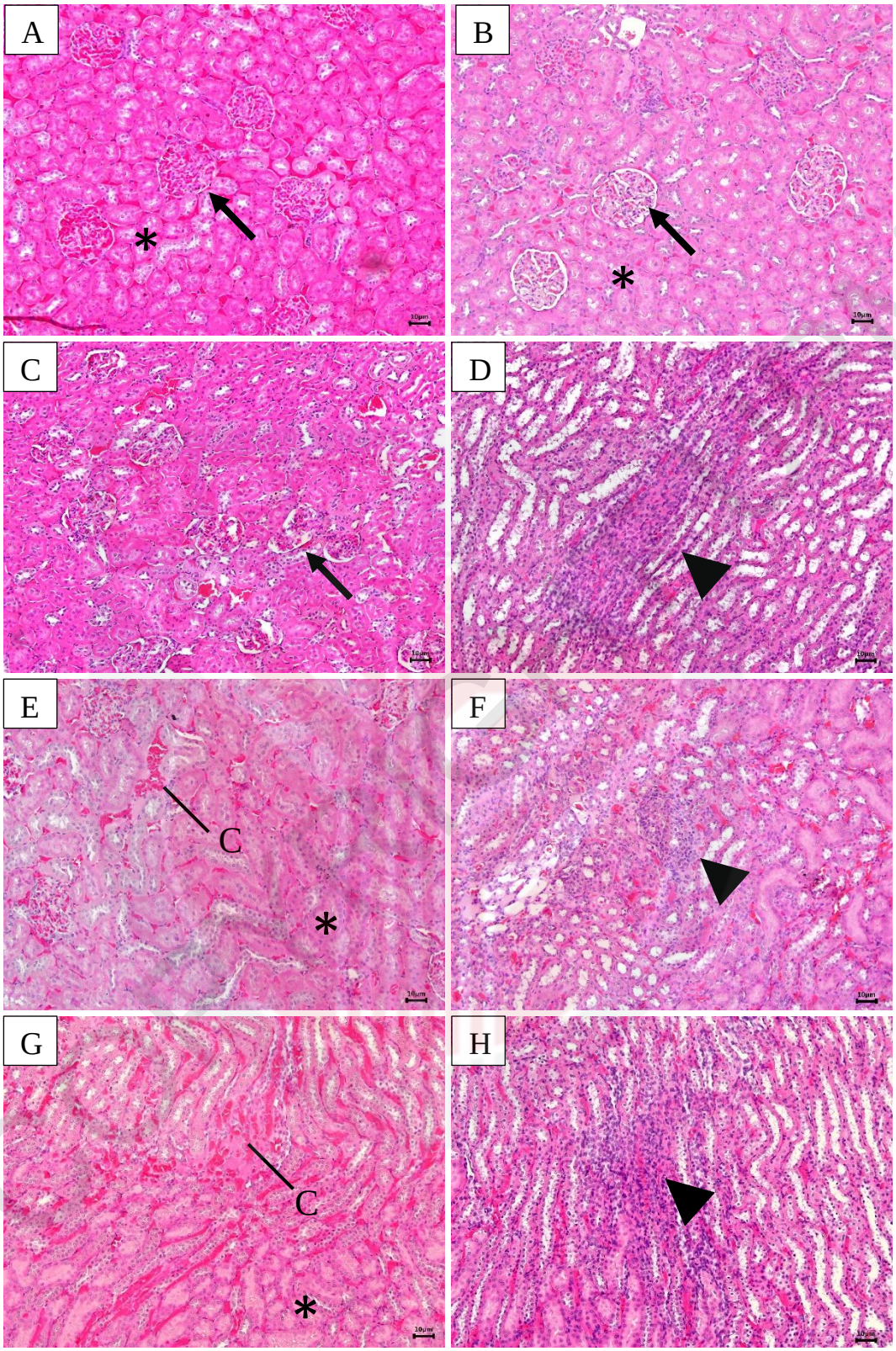


Figure 7. Photomicrograph of kidney sections of control, BPA, DMH and BPA-DMH treated groups. (A-B): Sections of a control kidney showing normal renal architecture with normal glomeruli (arrow) and tubules (asterisk). (C-D): Sections of BPA treated groups showing distorted glomeruli (arrow) and infiltration of inflammatory cells (arrowhead). (E-F): Sections of a DMH treated kidney showing tubular congestion (C), tubular dilation (asterisk) and focal infiltration of inflammatory cells (arrowhead). (G-H): Sections of BPA-DMH treated kidney showing tubular

congestion (C), tubular dilation (asterisk) and focal inflammatory cells infiltration (arrowhead). (Magnification 100X).

Table 7. Treatment groups vs histopathological scoring (crosstabs)

Group		Histopathological Scoring					
		0	**1**	**1.5**	**2**	**2.5**	**Total**
Control	Count	6 (100%)	0	0	0	0	6 (100%)
BPA	Count	0	2 (33.3%)	4 (66.7%)	0	0	6 (100%)
DMH	Count	0	1 (16.7%)	5 (83.3%)	0	0	6 (100%)
BPA-DMH	Count	0	0	2 (33.3%)	4 (66.7%)	0	6 (100%)
Total	Count	6 (20.8%	3 (12.5%)	11 (45.8%)	4 (16.7%)	0	24 (100%

In addition, BPA-DMH group is statistically significant different in histopathological changes as compared to BPA and DMH ($p < 0.05$) where the animals in BPA-DMH group (1.83 ± 0.26) exhibit moderate histopathological scoring in the form of moderate infiltration of inflammatory cells, intertubular congestion and dilated tubules (66.7%) compared to BPA (1.33 ± 0.26) and DMH (1.42 ± 0.2). The pathological changes in the group exposed to only BPA occur in the form of mild to moderate diminished and distorted glomeruli, infiltration of inflammatory cells and dilated tubules (66.7%). In some cases, the presence of congestion can also be observed in the BPA group, as illustrated in Figure 7. On the other hand, majority animals in the DMH group only show mild changes in renal architecture features in the form of congestion, diminished and distorted glomeruli, dilated tubules and infiltration of inflammatory cells (83.3%) as seen in Figure 7.

Lastly, there was no statistically significant different in histopathological changes between BPA and DMH ($p < 0.05$).

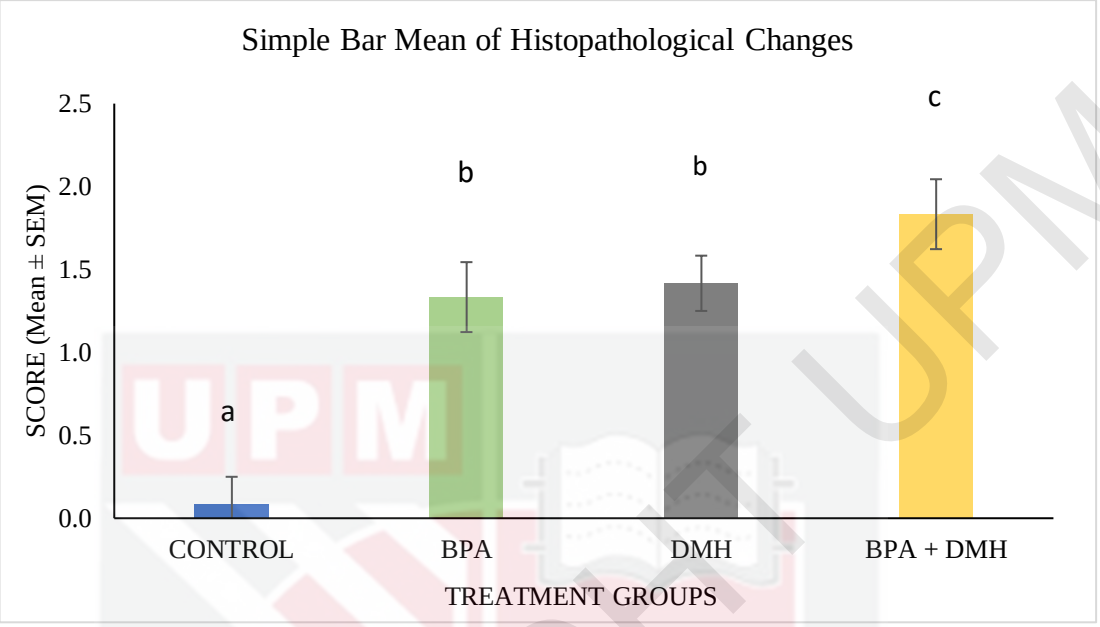


Figure 8. The bar graph shows the scoring of pathological changes in the kidney. The different treatments given for different groups showed different pathological changes scoring compared to the control. Results were expressed in mean ± S.E.M of three independent animals used per group in the study. Different alphabets represent $p \leq 0.05$ significantly different from BPA and DMH groups.

4.2. Spleen

4.2.1. Metastasis

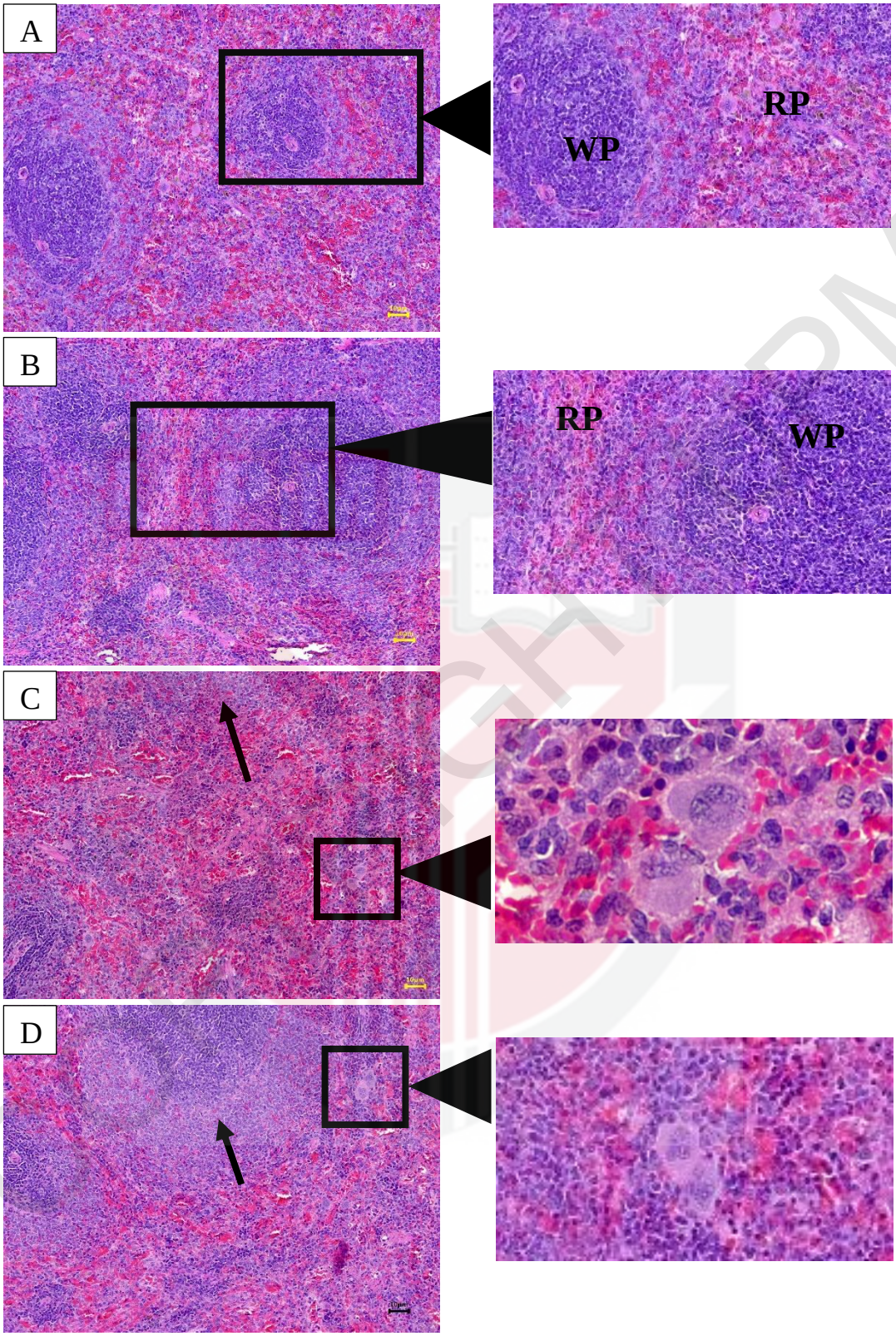
No metastatic cells from colorectal has been observed in the spleen of all treatment groups upon microscopic examination.

4.2.2. Histopathological Changes and Abnormalities

The treatment effects of BPA on the animals for any abnormalities on histopathology was performed. Data were analysed by using Pearson's Chi-Square

test. The statistical test has revealed statistically significant association between treatment groups and histopathological changes in the organ observed where majority of the treatment groups demonstrated typical histopathological abnormalities in spleen ($x(9) = 23.47, p < 0.05$) as displayed in Table 7.

The data was then presented as mean ± S.E.M. representing six independent animals utilised in each group in the experiment, as shown in Figure 12. The one-way ANOVA analysis on the histopathological changes or abnormalities in the kidney determined significant differences between groups ($F(3,20) = 28.96, p < 0.05$). A Tukey post hoc test revealed a statistically significant difference in histopathological changes in BPA and BPA-DMH as compared to control ($p < 0.05$). Table 7 shows that spleen histological features in the control group exhibit normal architecture of a well-defined spleen section (100%) as illustrated in Figure 9.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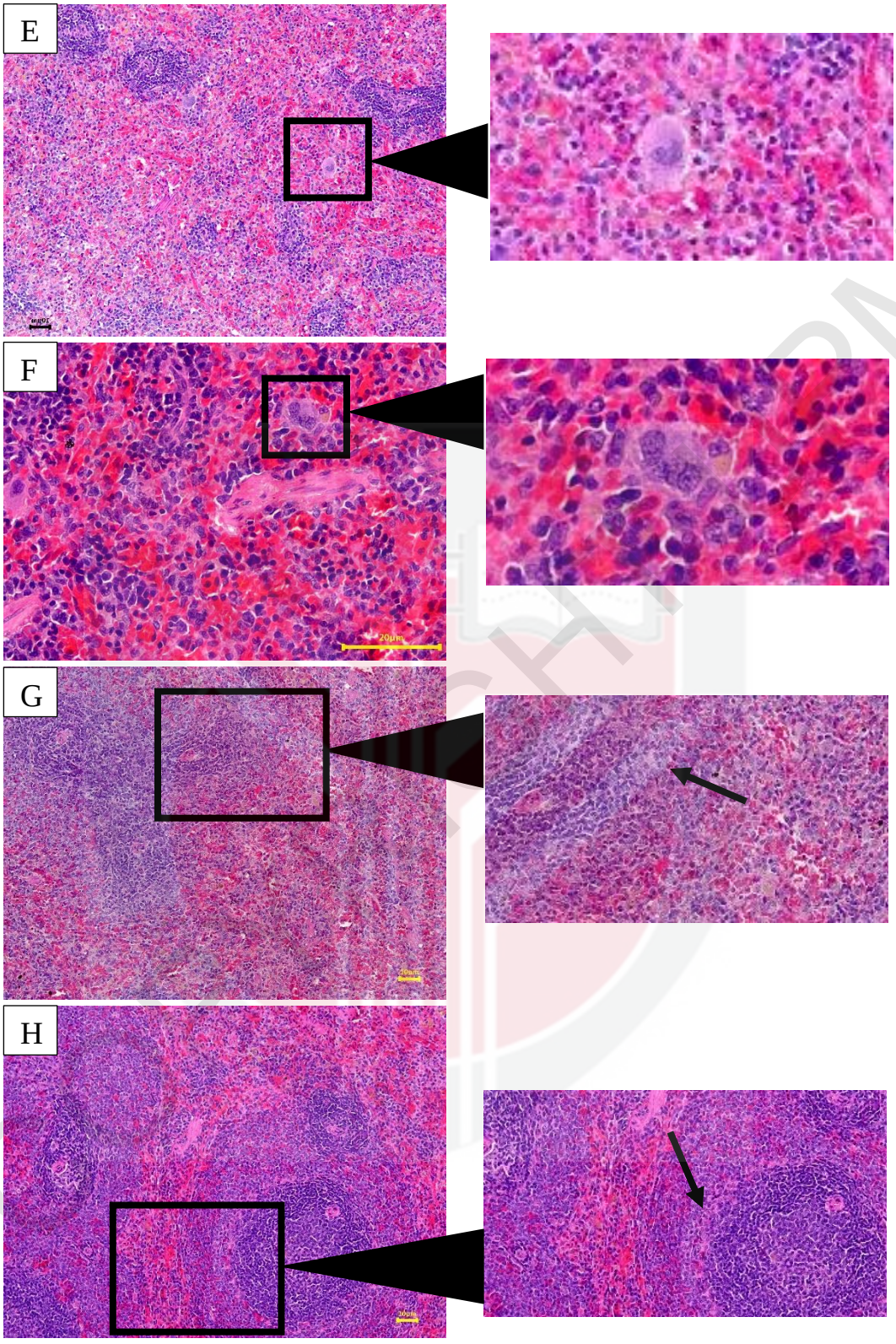

Figure 9. Photomicrograph of spleen sections of control, BPA, DMH and BPA-DMH treated groups. (A-B): Sections of a control spleen showing normal architecture of spleen with well-defined spleen sections of red pulp (RP) and white pulp (WP). (C-D): Sections of BPA treated group spleen showing reduced periarteriolar lymphoid sheath (PALS) cellularity (arrow) and presence of lymphocytes in the zoom in picture. (E-F): Sections of DMH treated group spleen

showing presence of lymphocytes in the zoom in pictures. (G-H): Sections of BPA-DMH treated group spleen showing reduced (PALS) cellularity (arrow). (Magnification 100X and 200X).

Table 8. Treatment groups vs histopathological scoring (crosstabs)

Group		Histopathological Scoring					
		0	**1**	**1.5**	**2**	**2.5**	**Total**
Control	Count	6 (100%)	0	0	0	0	6 (100%)
BPA	Count	0	4 (66.7%)	2 (33.3%)	0	0	6 (100%)
DMH	Count	5 (83.3%)	1 (16.7%)	0	0	0	6 (100%)
BPA-DMH	Count	0	4 (66.7%)	2 (33.3%)	0	0	6 (100%)
Total	Count	11 (41.7%)	9 (37.5%)	4 (16.7%)	0	0	24 (100%)

In addition to that, DMH group is statistically significantly different in histopathological changes as compared to BPA and BPA-DMH ($p < 0.05$) groups where the majority animals in DMH (0.25 ± 0.42) shows presence of lymphocytes but appeared normal in terms of spleen histological features (66.7%) as compared to BPA group (1.17 ± 0.26) which exhibit mild histopathological scoring as detailed in Figure 9. Table 7 displayed the altered histological features in group administered with BPA (66.7%) mainly appeared with presence of lymphocyte and mild decreased periarteriolar lymphoid sheath (PALS) cellularity. Apart from that, majority animal in BPA-DMH (1.17 ± 0.26) demonstrated mild features of spleen histopathological abnormalities (66.7%) where mild decreased (PALS) cellularity was observed as

shown in Figure 9. There was no statistically significant different in histopathological changes between control and DMH ($p > 0.05$). Lastly, histopathological changes also not statistically significant different between BPA and BPA-DMH treated group ($p > 0.05$).

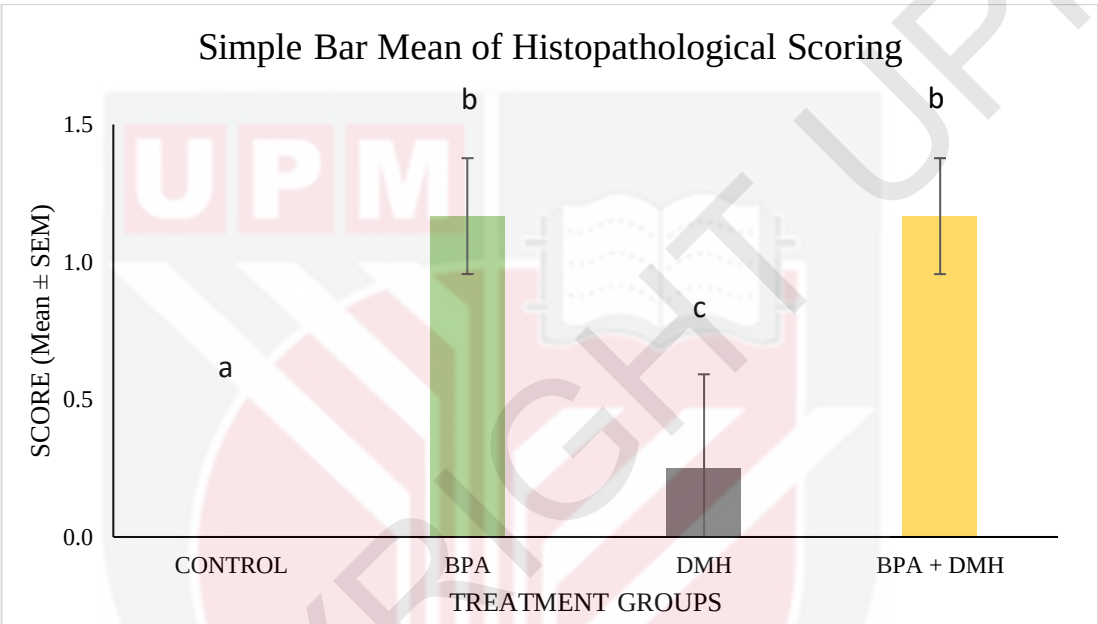

Figure 10. The bar graph shows the scoring of pathological changes in spleen. The different treatments given for different groups showed different pathological changes scoring compared to the control. Results were expressed in mean ± S.E.M of three independent animals used per group in the study. Different alphabets represent $p \leq 0.05$ significantly different from the BPA and BPA-DMH groups.

CHAPTER 5

DISCUSSION

5.1. Introduction

Bisphenol A (BPA), an artificial chemical that has been classified as an endocrine disruptor, has been widely used in the production of daily products, including bottles, pipes, and cans (Gao et al., 2015). Numerous studies have demonstrated that BPA disrupts the endocrine system by interacting with numerous endogenous biological receptors, such as oestrogen receptors (ER), androgen receptors (AR), and thyroid hormone receptors (THR). Consequently, the endocrine-disrupting effect of the synthetic compound would affect the normal function of various human body systems, including reproductive, nervous, metabolic, immune, and developmental systems (EFSA Panel on Food Contact Materials & Aids, 2015; ANSES, 2013). Recent studies indicating an association between BPA levels and the development of certain types of cancer, namely prostate, breast, and lung cancer, have also been a focus of research (Tse et al., 2017; Wang et al., 2017; Zhang et al., 2014).

It has long been known that exposure to BPA at high doses can affect the normal function of endogenous ER ligands, but numerous recent studies have demonstrated that even low-dose animal experiments can lead to various health problems (Vom Saal & Vandenberg, 2021). As previously mentioned, the LOAEL of BPA for oral administration has been determined to be 50 mg/kg/day, which is at least twice the dose used in the present study. However, according to the FDA, the TDI of BPA, which is defined as any doses lower than the LOAEL that can still be considered "safe doses"

and assumed not to cause adverse effects upon daily oral administration, has been set at 50µg/kg/day.

Interestingly, Khan et al. (2021) discovered that oestrogens and their similar structure and receptors play a crucial role in initiating and advancing CRC and other intestinal-related diseases. Because BPA can interact with ER, research has uncovered evidence of the effects of artificial compounds on CRC. Observation of spindle mesenchymal morphology has revealed, as mentioned previously, that BPA can induce EMT in colorectal cancer cells (Z. J. Chen et al., 2015).

However, *in vitro* study on CRC metastasis due to BPA exposure is lacking. Thus, determining the impact of BPA on CRC in an *in vivo* model is critical to demonstrating the occurrence of metastasis. Apart from that, the present study is also relevant to assessing the hazards of oral ingestion of low-dose BPA below NOAEL on hepatic, liver and kidney tissue architecture of CRC model.

5.2. Metastasis of Colorectal Cancer to Multiorgan

According to Z. J. Chen et al. (2015), protein modulation has contributed to the occurrence of EMT in SW80 cells exposed to BPA at high oral concentrations. The authors further justify that E-Cad expression has significantly declined after 96 hours of treatment of BPA, whereas, Vim expression in HCT-116 cells is surging. The results are indicative of the occurrence of EMT, which is regarded as the initial step in cancer cell invasion and metastasis of neighbouring cells.

However, a light microscopic examination of any organ samples in this study revealed no indication of the presence of metastasis cells. It has been reported that despite the advantages of CRC induced by DMH in rodents being identical to human

colon cancer, the downside of this method is that it requires multiple times injections to induce colon tumours as well as at least half a year of latency period (Nascimento-Gonçalves et al., 2021). The authors further justify that no metastasis to the liver finding has been reported in the DMH-induced colon tumours model. Therefore, a longer duration of CRC induction with DMH is required, as is a long latency period to observe the progression of the tumour.

Additionally, olive oil used to dissolve BPA before administration to animals contains antitumor phenols (Borzì et al., 2019). The authors further explain that olive oil polyphenols can inhibit the progression of colorectal cancer by reducing oxidative DNA damage. Numerous studies have also demonstrated that hydroxytyrosol (HT), one of olive oil's most important complex phenols, possesses anti-inflammatory and antitumor properties (Bernini et al., 2013; Richard et al., 2011). Thus, olive oil may contribute to the inhibition of CRC progression, rendering metastasis virtually impossible.

5.3. The histopathological changes of liver

Present study demonstrated that low-dose BPA administration to SD rats mainly induces moderate hepatic steatosis, providing strong evidence that there is no association of risk cancer with low-dose BPA exposure. In contrast, Weinhouse et al. (2014) revealed that neonatal mice subjected to chronic exposure of BPA developed neoplastic and pre-neoplastic lesions in the liver after 10 months. Specifically, the animals appeared with the presence of hyperplastic nodule surrounding the bile duct, which is not present in this study due to the short treatment period. Therefore, it can be concluded that sub-chronic exposure to low-dose BPA induces no tumours in hepatic tissue in SD rats.

Liver tissue, a known non-reproductive oestrogen target, expresses ERs that could theoretically interact with BPA (Khan et al., 2021b). Apart from that, BPA has also been determined to be metabolised in the liver through glucuronidation and sulfonation (Yalcin et al., 2016). Therefore, chronic exposure to the aforementioned synthetic chemical at relevant doses alters the regular expression of genes involved in hepatic lipid metabolism, resulting in the development of metabolic disorders (Ke et al., 2016). Additionally, previous studies have shown that gestational BPA exposure causes an abundance of fatty liver organs (Long et al., 2021). As in Martella et al. (2016), BPA has been reported to cause hepatis steatosis.

Similarly, this study found that BPA and BPA-DMH groups exhibited moderate steatosis-related histopathological changes. Hepatic steatosis is defined as intrahepatic fat comprising at least 5% of the liver mass (Nassir et al., 2015). The authors further highlighted that a small number of triacylglycerols accumulated in the liver could be hepatoprotective, but long-term lipid accumulation in the organ can cause metabolic disorder, inflammation, and also severe form of non-alcoholic fatty liver disease (NAFLD). Therefore, if steatosis persists, liver cirrhosis will develop later, increasing the likelihood of hepatocellular carcinoma (HCC) (Paternostro et al., 2021).

All in all, the study has revealed that sub-chronic low-dose BPA administration mainly mediates steatosis in the liver of CRC model with other mild histopathological changes but does not trigger the progression or metastasis of the aforementioned cancer to the organ.

5.4. The histopathological changes in kidney

The primary issue challenged in this study is whether low-dose BPA exposure could trigger renal tumours and progression to metastasis. The present study demonstrated that repeated exposure to low-dose BPA for 20 weeks negatively impacted kidney histopathology, which may lead to a decline in normal renal function.

The light microscopic studies demonstrated that the renal in the group treated with BPA and both BPA-DMH appeared with glomerular abnormalities and renal lesions, including inflammatory cells infiltration and tubular dilation, compared to the control group. Present findings are in agreement with the study by Priego et al. (2021) in which the negative effects of BPA exposure in the kidney were found to lead to renal function impairment. According to Kobroob et al. (2018), impaired tubular function and glomerular filtration can gradually diminish the organ's capacity to eliminate waste. It has been revealed by previous studies that blood BPA concentrations are elevated in patients with chronic kidney disease (CKD) (Palladino & Sereni, 2017). The authors further justified that patients with impaired kidney function produced only a small amount of urine, which led to the accumulation of the synthetic chemical in the body. Taken together, the present study shows that sub-chronic exposure to low-dose BPA leads to a range of histopathological changes in renal architecture features, which may gradually lead to deterioration of renal function, causing the BPA waste products to accumulate in the body and later exhibit toxic effects.

The results have shown that repeated exposure of low-dose BPA in group with DMH-induced CRC appeared with moderate histopathological changes compared to the other treatment groups. According to Velciov et al. (2013), some CRC patients have been determined to be associated with renal function reduction, which its underlying mechanism yet to be discovered indicates that sub-chronic exposure to low-

dose BPA does not affect progression or metastasis of CRC but indeed deteriorate the kidney normal architecture compared to other groups.

5.5. The histopathological changes in spleen

The present study indicates that no significant histopathological changes were observed in spleen tissue for groups treated with BPA. This result is consistent with Aydemir et al. (2020) who discovered that synthetic chemicals do not disrupt the typical tissue architecture but reduce the presence of CD30 and CD20 positive lymphocytes when administered 30mg/kg BPA.

Apart from that, the presence of lymphocytes was observed in BPA-only and DMH-only treated groups. This finding is in line with the study by Mohamed and Bastwrous (2021) that demonstrated various sizes of lymphocyte aggregation consisting of irregular dense chromatin and heterochromatic nuclei in groups treated with BPA. This finding may indicate that BPA exhibits toxic effects even at a low-dose, as Aydemir et al. (2020) reported. However, according to Basit et al. (2020), sub-chronic exposure to BPA has been determined to severely affect spleen tissue, as evidenced by red pulp dilation.

Besides, most of the spleen in the BPA and BPA-DMH treated group only appeared with mild decreased periarteriolar lymphoid sheath (PALS) cellularity. Histologically, the spleen consists of different lymphocytes encircling the central artery, which branches up from the reticular artery. PALS, which contains T cells predominantly, is one of the lymphocyte areas surrounding the central artery. Interestingly, the findings agree with a study by Gear and Belcher (2017) who successfully demonstrated severe alterations in the number, size, and cellularity of

PALS after 14 weeks of exposure to 40mg/kg BPA. The findings indicate that BPA administration has a dose-dependent negative effect on the PALS region.

All in all, the present study revealed that low-dose BPA does not deteriorate spleen architecture in CRC model as BPA-only treated group was not significantly different in histological changes as compared to BPA-DMH group.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1. Summary

Bisphenol A (BPA) has been exposed daily to humans mainly through ingestion as the chemical can leach from various daily life products, commonly plastic bottles, and food containers. It has been known for BPA to act as a carcinogen to increase *in vivo* tumorigenesis and progression of colorectal cancer (CRC) by inducing epithelial-to-mesenchymal transitions (EMT). However, *in vivo* progression and metastasis of cancer mentioned above to surrounding organs has not been fully discovered and elaborated on in previous studies. Thus, this study was carried out to determine the effects of BPA on *in vivo* metastasis of CRC to surrounding organs through histopathological analysis. Sub-chronic exposure to low-dose BPA was found to not trigger the progression of CRC cells to the liver, kidney, and spleen but altered their regular tissue architectures features. Specifically, BPA deteriorates standard renal architecture in CRC model compared to other treatment groups.

6.2. Conclusion

Conclusively, the present study provides primary evidence that sub-chronic exposure to low-dose BPA does not trigger the progression of colorectal cancer cells to the liver, kidney, and spleen but alters the regular tissue architectures features of the organs mentioned above. This finding further strengthens the speculations that low-dose BPA may exhibit toxic effects on multiorgan despite being considered “safe doses.” The present study will highlight an essential concern about human health due

to the toxicity of bisphenol exposure from daily products. Nonetheless, the effects of BPA exposure should be further dissected in the future to comprehend the mechanisms underlying the toxic effects exhibited to multiorgan and its potential to trigger the proliferation and progression of CRC cells to surrounding organs.

6.3. Future Recommendation

Current study has only managed to show the effects of BPA exposure on histological features of multiorgan due to the short DMH induction period which is insufficient to observe for tumours progression in colorectal. Notably, CRC induced by DMH in rodents requires multiple times of injection to induce colon tumours as well as at least half year of latency period (Nascimento-Gonçalves et al., 2021). Thus, it is recommended to increase the DMH induction and latency period to observe the development of tumours in the colon. Apart from that, further study on prolonged BPA treatment period also recommended to be further investigated to understand the potential role of its chronic exposure on colorectal cancer cells growth and migration.

Other than that, the BPA concentration utilised in this study may not reflect the real daily amount consumed by human in general. Therefore, BPA concentration used in future study must accurately imitate the daily amount of BPA consumption to establish the toxic effects of BPA which potentially leads to morbidity.

Apart from that, olive oil which has been used to dissolve BPA should be replaced with other alternatives solvents to dissolve the BPA such as alcohol as the man-made compounds is highly soluble in oxygen-containing solvents including carbonyl and hydroxyl groups (Sun et al., 2020). Other than that, BPA can also be dissolved in

dimethyl sulfoxide (DMSO), a commonly used solvent (Dimethyl Sulfoxide - American Chemical Society, n.d.).

As this study only covers the superficial of the issue, detailed understanding on the underlying mechanism at cell and molecular level involved in the toxic effects of BPA to the surrounding organs are needed.

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APPENDICES

APPENDIX A: PEARSON'S CHI SQUARE STATISTICAL ANALYSIS

1) Chi-Square Test of liver

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	33.600	9	.000	.000	
Likelihood Ration	37.264	9	.000	.000	
Linear-by-Linear Association	9.967	1	.002	.001	.000
N of valid cases	24				

2) Chi-Square Test of Kidney

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	29.333	9	.001	.000	
Likelihood Ration	34.630	9	.000	.000	
Linear-by-Linear Association	8.849	1	.003	.002	.001
N of valid cases	24				

3) Chi-Square Test of Spleen

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	23.467	9	.005	.001	
Likelihood Ration	30.167	9	.000	.000	
Linear-by-Linear Association	5.589	1	.018	.020	.010
N of valid cases	24				

APPENDIX B: ONE-WAY ANOVA STATISTICAL ANALYSIS

1) Histopathological changes in liver

Table 9. Statistical analysis of histopathological changes in liver by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Between groups	11.167	3	3.722	40.606	P < 0.05
Within column	1.833	20	0.092		
Total	13.00	23			

Table 10. Statistical analysis of histopathological changes in liver between group comparison by Tukey's honestly significant difference (HSD) post hoc test.

Treatment Groups	Mean difference	Standard error	95% CI differences	P value
Control - BPA	1.6667	0.1748	-2.156 to -1.177	p < 0.05
Control - DMH	1.0000	0.1748	-1.489 to -1.511	p < 0.05
Control - BPA+DMH	1.6667	0.1748	-2.156 to -1.177	p < 0.05
BPA - DMH	0.6667	0.1748	0.177 to 1.156	p < 0.05
BPA - BPA+DMH	0.0000	0.1748	-0.489 to 0.489	p > 0.05
DMH - BPA+DMH	0.0667	0.1748	-1.156 to -0.177	p < 0.05

2) Histopathological changes in kidney

Table 11. Statistical analysis of histopathological changes in kidney by one-way ANOVA

Treatment Groups	Mean difference	Standard error	95% CI differences	P value
Between groups	9.125	3	3.042	P < 0.05
Within column	2.333	20	0.117	
Total	11.458	23		

Table 12. Statistical analysis of histopathological changes in kidney between group comparison by Tukey's honestly significant difference (HSD) post hoc test.

Treatment Groups	Mean difference	Standard error	95% CI differences	P value
Control - BPA	-1.5000	0.1972	-2.052 to -0.948	p < 0.05
Control - DMH	-0.8333	0.1972	-1.385 to -0.281	p < 0.05
Control - BPA+DMH	-1.5000	0.1972	-2.052 to -0.948	p < 0.05
BPA - DMH	0.6667	0.1972	0.115 to 1.219	p < 0.05
BPA - BPA+DMH	0.0000	0.1972	-0.552 t0 0.552	p > 0.05
DMH - BPA+DMH	-0.6667	0.1972	-1.219 to -1.115	p < 0.05

3) Histopathological changes in spleen

Table 13. Statistical analysis of histopathological changes in spleen by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Between groups	6.698	3	2.233	28.964	P < 0.05
Within column	1.542	20	0.077		
Total	8.240	23			

Table 14. Statistical analysis of histopathological changes in spleen between group comparison by Tukey's honestly significant difference (HSD) post hoc test.

Treatment Groups	Mean difference	Standard error	95% CI differences	P value
Control - BPA	1.1667	0.1603	-1.615 to -0.718	p < 0.05
Control - DMH	0.2500	0.1603	-.699 to 0.199	p > 0.05
Control - BPA+DMH	1.1667	0.1603	-1.615 to -0.718	p < 0.05
BPA - DMH	0.9167	0.1603	0.468 to 1.365	p < 0.05
BPA - BPA+DMH	0.0000	0.1603	-0.449 to 0.449	p > 0.05
DMH - BPA+DMH	0.9167	0.1603	-1.365 to -0.468	p < 0.05