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Research Article

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Sexual dimorphism of colorectal cancer in humans and colorectal tumors in a murine model

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Abstract:

**Background:** Sexual dimorphism (SD) is the difference in morphology and physiology between sexes of the same species; in diseases, SD can reflect the susceptibility associated with gender in humans. In colorectal cancer, men have a higher incidence than women, independently between ethnicity or geographical location, suggesting that sex steroids are involved in the development of colorectal cancer.

**Methods:** We determined sex, as a risk factor for colorectal cancer from the GLOBOCAN database; Then, we used induction of colorectal tumors by azoxymethane and dextran sulfate of sodium treatment as the experimental strategy in males and females mice; also we gonadectomized independent males and females animals. Finally, we determined in vitro proliferation of a human cell line HCT116 exposed to estradiol, testosterone or dihydrottestosterone.
**Results:** Sex as a risk factor for colorectal cancer showed clear and statistically significant susceptibility of men in Mexico and worldwide. In the murine model of colorectal tumors, males developed more and larger tumors than females. Further analysis showed that ovariectomized females developed more tumors in number, but all about the same size. Meanwhile gonadectomized males had fewer tumors in number and smaller in size. Surprisingly, only estradiol showed an effect in vitro on the proliferation index on human cell lines of colorectal cancer.

**Conclusions:** Men showed enhanced susceptibility to develop colorectal cancer than females; in the animal model, male mice presented augmented development of colorectal tumors, which was reverted in gonadectomized male mice, female mice increased the number of tumors, the above suggests that androgens have a crucial role in explaining sexual dimorphism in the incidence of colorectal cancer. Estradiol diminished the in vitro proliferation of HCT-116 colon cancer cell line, opposite, there was no change of in vitro proliferation on cells exposed to testosterone or dihydrotestosterone, therefore, the effect in vitro could be by their interaction with other cells or systems.

**Keywords:** Colorectal cancer, sexual dimorphism, sex steroids serum levels, colorectal tumors, male, female.

**Graphical Abstract:**
Sexual dimorphism (SD) is the difference in morphology and physiology between sexes of the same species. Particularly in the study of diseases, SD reflects in the susceptibility associated with sex, having impact on mortality, morbidity, prevalence, intensity, severity, behavioral changes, and immune response\(^1\). For example, colorectal cancer (CCR) is the third most common heterogeneous neoplasm in the world, in which several studies have reported that men have a higher incidence than women. Sexual dimorphism can be interpreted by multiple causes, which can be grouped in: gender and sex categories, including differences in behavior and physiological conditions. Moreover, sexual
dimorphism is independently maintained between ethnicity or geographical location, suggesting that intrinsic factors play a fundamental role to explain it.\textsuperscript{3}

The concentration of sex steroids is one of the most important intrinsic factors that differ between both sexes; these molecules have important roles in the gut: Estrogens influence epithelial membrane permeability, serotonin production, expression of tight junctions, inflammation, and microbiome composition; Androgens are poorly understood; however, in a mouse model alters the proliferation of enterocytes and increases the size of crypts. Further in vitro studies with colon cancer cell lines such as DLD-1, HCT-116, SW480, and CaCO2 have shown that estradiol reduces the viability of these cells, and testosterone (T4) and dihydrotestosterone (DHT) increase the apoptosis in CaCO2 and HCT116.\textsuperscript{15}

There are few in vivo studies in which the sexual dimorphism is clear; Lee et al., described a rapid and effective method for modeling human colitis-associated cancer using the carcinogen azoxymethane (AOM) and subsequently treated with the pro-inflammatory agent dextran sulfate sodium (DSS) on ICR mice. In this study they observed that male mice developed more tumors than females; however, the role of sex steroids is unclear. In the Apc\textsuperscript{Pirc/+} (Pirc) rat model of early colonic cancer, male Pirc rats developed twice as many adenomas than females; here, neither ovariectomy nor reconstitution with estradiol in PIRC rats affects the development of adenomas. Contrarily, Song et al, showed the protective effect of endogenous 17-β Estradiol (E2) or the E2 replacement in ovariectomized female ICR mice against AOM/DSS-induced colitis.\textsuperscript{18,19}
In the AXO-DSS model in C57BL6 mice, testosterone strongly enhances the induction of colorectal cancer\(^{20}\); consistently, the orchiectomy in PIRC rats and C57BL6 mice reduce drastically the number of tumors and this effect is countered with the reconstitution with DHT, by contrast the treatment with testosterone reduce significantly tumor incidence induced by dimethylhydrazine and Dextran Sulphate of Sodium (DSS) in Balb/c mice\(^{17}\). These apparently contradictory results shows the importance of proposing a study with homogenized conditions that evaluated the participation of sex steroids in the induction of colorectal tumors in males and females. Therefore the goal of this research is to determine, if sex-related susceptibility in the induction of colorectal tumors is mediated by the concentration of the principal sex steroids (E2, T4 and DHT), evaluated in a murine model.

**Methods**

*Evaluation of sex as a risk factor in Mexico and worldwide.*

The most recent data (2020) was obtained from the GLOBOCAN database\(^2\). The parameters chosen were: men and women over 45 years old worldwide, particularly in Mexico. With this data a statistical analysis was carried out (risk factor test & confidence interval).

*Animals and ethics statement*

Animals care and experimental practice were conducted at Unidad de Modelos Biológicos (UMB) in the Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autonoma de México (UNAM). All experimental procedures in the animals were approved by the Institutional Care and Animal Use Committee (CICUAL), ID Number 6298, adhering to Mexican regulation (NOM-062-ZOO-1999), and in
accordance with the recommendation from the National Institute of Health (NIH) of the United States of America (Guide for the Care and Use of Laboratory Animals).

Mice of the syngeneic strain Balb/c AnN were purchased from Harlan, Facultad de Química, UNAM, México. The animals were housed at the UMB at a controlled temperature (22°C) and 12 hours of light-dark cycles.

*Gonadectomy in male and female mice*

Gonadectomies were performed at four weeks of age. Balb/c mice according to the protocol previously reported by Villavicencio, et al, 21. Briefly, animals were anesthetized with a cocktail of xylazine and ketamine (Pisa, Atitalaquia Hidalgo, México). Ovariectomy (OVX) was carried out by making an incision in the dorsal part; the ovary was located under the muscle layer and a cut was made to enter the peritoneal cavity. The oviducts were ligated and the ovary was removed. After removal, the incision was closed using an absorbable synthetic suture (triple zero polyglycolic acid) (Atramat, México).

For orchiectomy (ORX), an incision was performed on the ventral part of the scrotum. The testicular fat was located and gently pulled away exposing the epididymis, vas and blood deferents, and testis. The blood vessels were ligated and the testicle was removed. Once both testicles were removed, the surgery was concluded by suturing the skin using an absorbable synthetic suture.

*Colon tumor induction*
The induction of colorectal tumors was performed following the protocol previously reported by Leon-Cabrera \textsuperscript{22}. Briefly, four weeks after gonadectomy, male and female mice: control, sham, and gonadectomized were injected intraperitoneally with 12.5 mg/kg of azoxymethane (AOM) (Sigma-Aldrich, St. Louis, Missouri, USA). Five days later, mice were treated with the pro-inflammatory agent dextran sulfate sodium DSS (Alfa Aesar, Ward Hill, Massachusetts, USA) in water ad libitum for seven days. Subsequently, two weeks of rest (with potable water), and the cycle was repeated three times.

\textit{Sacrifice and tumor processing}

At day 70 from the start of AOM-DSS treatment, mice were euthanized by inhalation of a mixture of air and sevoflurane (5\%) and cervical dislocation. Blood was drawn by cardiac puncture to obtain the serum. Subsequently, the colon of each mouse was removed and cut to expose the tumors and placed on graph paper. First, we counted the total number of tumors then, we evaluated the size by determining tumors larger than two millimeters.

\textit{Quantification of concentration of sex steroids}

The sex steroids were extracted by an ether-methanol method, and the concentration was calculated using an ELISA kit for E2, T4 (Arbor Assays, Ann Arbor, Michigan, USA), and DHT (Eagle Bioscience, Nashua, New Hampshire, USA) according to manufacturer’s protocol.

\textit{Analysis of proliferation in colon cancer cell lines}

The HCT-116 cell line was cultured in RPMI 1640 medium (Sigma, St. Louis, Missouri, USA) supplemented with 10\% of FBS, glutamine, antibiotics, sodium pyruvate, and essential amino acids.
(GIBCO, Invitrogen, Grand Island, New York, United States). Cells were counted in a Neubauer Chamber and placed in 96-well culture plate at 1500 cells/well cultured with RPMI free of sex steroids and phenol red for 24 hours. Subsequently, increasing concentration of E2, T4, and DHT from $10^{-9}$ to $10^{-5}$ M was added to corresponding wells. After 48 additional hours, the proliferation was determined with CyQUANT KIT following the manufacturer’s protocol in a Cytation 1 Cell Imaging Multi-Mode Reader (Biotek, Santa Clara CA, United States).

**Statistical analysis**

To evaluate sex as a risk factor, the relative risk was calculated based on the data obtained from the GLOBOCAN database (2020). Then, confidence intervals were calculated to get the significance. Data from 2-3 independent experiments are charted as mean +/- standard deviation and analyzed with Prism 5 software (GraphPad Software Inc.). Thereafter, one-way ANOVA was performed, followed by a Tukey post-hoc test. The difference was considered significant when P< 0.05.
Results

*Evaluation of sex as a risk factor in Mexico and worldwide.*

The incidence of colorectal cancer was obtained from the most recent data available from GLOBOCAN (2020). Worldwide men have a higher relative risk (1.47) to develop colorectal cancer. In Mexico, men remain more susceptible with a relative risk of 1.37 (p<0.05). This analysis is the first to confirm with a statistical analysis that sex is a risk factor to develop colorectal cancer in Mexico and worldwide (Figure 1).

**Figure 1.** Analysis of sex as a risk factor to develop colorectal cancer in the world and Mexico. Men have a significative relative risk both in the world and in Mexico. * P<0.05. ♀ Women, ♂ Men.
**Tumor number in the murine model**

The tumors were induced by AOM-DSS in all experimental groups. Male had a statistically significantly higher number of tumors than females. Interestingly, the gonadectomy in female mice increases the number of tumors; while in male had the opposite effect reducing the number. The above suggests a possible protection role of estrogens and a protumoral role of androgens (Figure 2).

**Figure 2.** Representative photos (A) and statistical analysis of the number of tumors (B). *** represent p< 0.05. Black arrows point to tumors. Ctrl, control; Gx, gonadectomized; Sham, simulated surgery. Female ♀ male ♂
Tumor size in the murine model

The size of tumors was evaluated by counting the tumors > 2 mm. In colorectal cancer development, the tumor size is a very important parameter since it is associated with tumor aggressivity. Our results showed that male had statistically larger tumors in size than females. Interestingly, the gonadectomy in female mice had no effect in the size of tumors, while in male reduce drastically the size of tumors. The above suggest that androgens have a more important role in the development of colorectal tumors (Figure 3).

Figure 3. Representative photos (A) and statistical analysis of the size of tumors (B). *** represent p< 0.05. Red circles represent the size of tumors. Ctrl, control; Gx, gonadectomized; Sham, simulated surgery. Female  male
Sex steroids concentration in serum

Sex steroids concentration was evaluated through ELISA kits. As expected, females had more concentration of estradiol than males. The serum estradiol levels in control females with tumor was significantly lower than control females, but remained slightly higher than males. Unexpectedly, gonadectomized females had no change in the concentration of estradiol compared to control females, but remained higher than gonadectomized males.

Testosterone level is higher in the serum of intact males than females. Nevertheless, in control mice and gonadectomized with tumor the serum concentration of testosterone decreased drastically. In the case of DHT, the intact male group presented a higher concentration compared to females. Predictably, gonadectomized males had a lower concentration of these hormones. Interestingly, the control female group had a higher concentration level of DHT compared to the intact female group; this effect was inhibited with ovariectomy, suggesting that estradiol is being converted to DHT in control females.
Figure 4. Concentration of principal sex steroids in all experimental groups; Estradiol E2 (A), Testosterone T4 (B) and Dihydrotestosterone DHT (C) ***p<0.05. Int, intact (mouse without tumor); Ctrl, control; Gx, gonadectomized; Sham, simulated surgery. Female ♀ male ♂

In vitro proliferation of colon cancer cell lines

The proliferation was determined with CyQuant kit, after 48 hours of exposure to HCT-116 cells with sex steroids (E2, T4, or DHT) or vehicle (ethanol). The treatment with estradiol significantly reduces the proliferation in a dose-dependent manner. Interestingly, neither T4 not DHT had an effect on cell proliferation.
Discussion

In humans, colorectal cancer (CRC) is one of the most common malignant neoplasms; it is ranked 2nd to 4th in terms of incidence, depending on the location, type of cancer or gender\textsuperscript{23}. The study of the risk factors is relevant, since the incidence is increasing in terms of morbidity and deaths. Many factors may be responsible for development of this disease; the progression from adenoma to carcinoma shows clear sexual dimorphism, with preferential male development of both adenomas and CRC\textsuperscript{17}. We decided to study the role of sex hormones in the progression of CRC using a murine model widely accepted by AOM-DSS method, using gonadectomy to evaluated the role of sex steroids.
As expected, the induction of tumors was dimorphic, males had more and larger tumors than females. In line, ICR, C57BL/6, and APC/min male mouse develop more tumors than females. Interestingly, in our results the gonadectomy in females reduce the number of neoplasias, and orchiectomy diminishes the number and the size of tumors in males. The above is particularly relevant, since the size of tumor in colorectal cancer is closely associated with the stage of cancer and aggressivity of tumor, suggesting that androgen have a major role in the progression of colorectal tumors compared to estrogens. In accordance, orchiectomy in rats that spontaneously are susceptibly to develop adenomas and C57BL/6 mice reduce the formation of neoplastic lesions, and the reconstitution with DHT inhibite this effect. In line, our results showed that DHT in Gx males (that develop fewer and smaller tumors than control) has a lower effect than in control male mice.

Supporting this idea, the expression of androgen receptor is not sex-associated; men with hypomethylation of androgen receptor have a higher risk of developing CCR; contrarily, the hypomethylation of this receptor is not associated with CCR in women. In line, propionate of testosterone has different effects on the histology of tumors and the expression of inflammation markers such as IL-6, IL-β, COX-2 in females compared with male animals treated with azoxymethane and dextran sulfate of sodium. Then, it is necessary to propose studies that address the difference in the effect of both estrogens and androgens in the physiology of male and female colon. An important observation is that the concentration of testosterone is lower in control males with tumors than in intact animals. In line, the level of T4 in serum in patients with CCR is lower compared with control cases.
One possible explanation of this phenomenon is that CAG sequence repeats on androgen receptor (commonly found in CCR patients) is highly associated with lower T4 by unknown mechanism\textsuperscript{30}. In the case of Gx males, the levels of estradiol are elevated, and this coincides with a lower level of T4 in this group. Although the mechanism is not clear, ORX animals produce more estradiol\textsuperscript{31}. This phenomenon could be due to the activation of the adrenal gland or the hyperactivity or overexpression of aromatase; further studies are needed to elucidate it.

In humans, men have more incidence of CCR than women\textsuperscript{2}. Historically, the difference in risk factors such as drinking alcohol, cigarette smoke, and diet was considered the cause of sex dimorphism; however, even adjusting dates and taking into account these factors, men still remain more susceptible. The above suggests that intrinsic factors play a fundamental role in the development of CCR. As mentioned, the concentration of sex steroids is the principal factor that differentiates between men and women\textsuperscript{3}. In the case of CCR, interestingly, the lower concentration of circulating testosterone is associated with overall survival, although, the expression of androgen receptors is directly associated with tumor size, tumor differentiation, and distant metastasis\textsuperscript{27,32}.

These observations open the possibility of using drugs that inhibit the interaction of androgens with its receptor, such as flutamide or apalutamide, which are drugs approved for the treatment of prostate cancer. In the case of estrogens, the hormonal therapy replacement (HTR) and the consumption of food rich in phytoestrogens reduce the risk of developing CCR in postmenopausal women. Regrettably, HTR is associated with a higher risk of suffering breast cancer\textsuperscript{33–35}. Estrogens
favor the proliferation and mutation of breast cancer cells by the interaction with the estrogen receptor alpha. Contrarily, the expression of estrogen receptor beta is directly associated with high survival and inversely with the stage of tumor in CCR patients. The above support the idea of promoting the use of specific agonists of estrogen receptor beta such as diarylpropinitrile that inhibit the viability of colon tumor cells in vitro.

The mechanism by which sex steroids participate in the development of CCR is poorly understood. However, estradiol inhibits directly the growth of colon cancer cells supporting our results. This inhibition is through ER-beta activation that crosstalk with the MAPK pathway or directly activates the gene transcription, stimulating genes that inhibit the proliferation or induce apoptosis, such as TGF-β. Although some studies (including this), have shown that androgens have a preponderant role in the development of CCR (mainly in males). The action mechanism by which they act is largely unknown. For example, T4 inhibits tumor growth by the activation of membrane androgen receptor.

Besides, in line with our work, in a recent study, the ORX reduces the number and size of tumors; however, the treatment with propionate of testosterone does not inhibit this effect, which suggests that other androgens (such as DHT) have the most import role in the growth of tumors. In line, the reconstitution with DHT on ORX PIRC rats, that develop fewer tumors than control, inhibits the effect of surgery in the induction of neoplasias. In our study, there is no effect on the progression of colon cancer cell lines treated with T4 or DHT, which suggests that the effect of these hormones could be by...
their interaction with other cells in the tumor microenvironment such as immune, stromal, nervous system cells, or microorganisms in the intestinal microbiota.

**Conclusions**

Statistically male are more susceptibles to develop colorectal cancer in Mexico and worldwide. In our mouse model, males showed augmented tumors in number and size, this effect were reverted in gonadectomized males. Regarding females, we found less tumors in number with smaller size compared to males; when this females were gonadectomized, tumors increased in number but not size. The finding that orchiectomized males present diminished tumors in number and size suggesting the important protumor role of androgens in the physiopathology of colorectal cancer. In ovariectomized females, the increase in number of tumors suggest a protector role of estradiol to prevent the development of colorectal tumors.

Supporting the protector role of estradiol, we found a decreased in the proliferation index of a human colon cancer cell line (HCT-116) exposed to this female hormone. However, we did not observe a change in the proliferation of this cell line exposed to Testosterone and dihydrotestosterone, suggesting that indirect modulation to develop colon tumors through other cells or components, such as immune or neuro systems.
**Ethics statement.**

Animals care and experimental practice were conducted at Unidad de Modelos Biológicos (UMB) in the Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autónoma de México (UNAM). All experimental procedures in the animals were approved by the Institutional Care and Animal Use Committee (CICUAL), ID Number 6298, adhering to Mexican regulation (NOM-062-ZOO-1999), and in accordance with the recommendation from the National Institute of Health (NIH) of the United States of America (Guide for the Care and Use of Laboratory Animals).

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets generated and analyzed during the current study are included in the present manuscript. Also, they are openly available from the corresponding author on request.

**Competing interests**

The authors declare that they have no competing interests

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**Authors' contributions**

JMM and LIT conceived and designed the research; YRS, VHDR and CAGC performed the research and acquired the data; MSM, KENC, CAGC analyzed and interpreted the data. All authors were involved in drafting and revising the manuscript.

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The datasets generated and analyzed during the current study are included in the present manuscript. Also, they are openly available from the corresponding author on request.

**References**


27. Albasri, A. M. & Elkablawy, M. A. Clinicopathological and prognostic significance of androgen receptor overexpression in colorectal cancer Experience from Al-Madinah Al-Munawarah, Saudi...


36. Barone, M. *et al.* ERβ expression in normal, adenomatous and carcinomatous tissues of patients