

Research Article

In vivo Protective Role of *Amaranthus viridis* Extract and Direct Folate Treatment on the Sperms of Swiss Albino Mice

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- *Amaranthus viridis* extract

Abstract

Background: Synthetic drugs are available for treating infertility in men caused by various reasons as in use of anti-folates, poor health and environment etc. Plant supplements are preferred over chemicals due to lesser side effects and replenishment of the body through natural dietary resources.

Methods: Albino mice (*Mus musculus*) have been used as animal models for studying the benefits of *Amaranthus* leaf extract (Soxhlet extraction) and folate on the methotrexate dampened sperm conditions affecting the male fertility in mice.

Results: Methotrexate-exposed mice with abnormal sperm morphology ($p=0.034$), reduced motility ($p<0.0001$), dead sperms ($p<0.0003$) indicated significant damage induced by methotrexate. Folate and *Amaranthus* treatment reduced damage ($p^{FA}=0.30$ and $p^{AE}=0.37$). Mice showed significantly improved mean values, and drastic improvement in sperm motility ($p^{FA} = 0.0053$, $p^{AE} = 0.0024$), reduction in dead sperms ($p^{FA} = 0.0059$ and $p^{AE} = 0.0092$), and no significant impact on the head morphology ($p^{FA} 0.38$, $p^{AE} = 0.15$).

Conclusion: In our study we have observed that Folate and *Amaranthus* extract have noticeably contributed to improvement of sperm morphology in male mice. Further analysis can be done for evaluating its potential in treating fertility related complications in men.

INTRODUCTION

Couples visiting fertility centers are treated for sperm abnormalities owing to the inability to conceive due to poor sperm count/quality. Identifying factors contributing to poor semen should be combined with adopting a healthy lifestyle to help in sperm health recovery and increased pregnancy chances. *Amaranthus viridis* and folate treatment was used in mice administered methotrexate known for its detrimental effects on fertility, to investigate their benefits in improving sperm quality. The idea was to identify an agent that can be easily incorporated into our diet that can improve sperm performance and benefit couples visiting fertility centers while planning a pregnancy.

In humans, a sperm count of 20 million/ml and above with more than 60% motility is considered adequate to confer a chance of pregnancy in the couple with a normal healthy female partner. In addition, the colour, fructose levels, pH, liquefaction are indicator tests for normal sperm quality. Low sperm count is an indication of decreased fertility as more and more germ cells

are lost during spermatogenesis [1,2]. Abnormal sperms may be due to external factors like kidney and liver disorders, unhealthy lifestyle and work atmosphere, smoking etc [3,4]. It is essential to evaluate sperm head morphology in sub-fertile men especially where the couple opts to use assisted reproductive techniques (ART) to conceive.

Studies in the past have associated higher folate levels with lower frequencies of abnormal sperms and depleted sperm count. Folic acid is a type of Vitamin B- B9 an essential nutritional requirement for the body as it participates in *de novo* synthesis of DNA in mammalian cells and is crucial to many metabolic pathways which include histidine cycle, serine and glycine cycle, methionine cycle. It also helps maintain erythropoiesis, manages homocysteine levels in blood, inter-conversion of amino acids and is essential in thymidylate and purine synthesis [5]. Zinc, a micronutrient, showed significant association with increased sperm concentration in sub-fertile males (18.5×10^6 cells/ml [$p=0.02$]) in a study conducted to evaluate the effects of folate and zinc on male fertility [6].

Deficiency in folic acid has been associated with decreased sperm count in mice. Folic acid deficiency can arise due to various reasons like cancer, poor dietary intake and use of anti-folates such as methotrexate which affects folic acid metabolism as it has a higher affinity for DHFR (Dihydrofolate reductase), resulting in irreversible inhibition of the enzyme [7].

A study by Young et al., found that lower dietary intake of antioxidants such as vitamin C and increased levels of oxidizing agents in semen have been associated with decreased motility, count, viability and abnormal morphology in both human and animal sperm [8].

Anti-folates affect all mammalian cells because they inhibit folate metabolism, for this reason they have been under scanner for causing possible damage to sperm cells in mammals [9]. Methotrexate being an anti-folate, is often used as a drug to treat diseases such as cancer and autoimmune diseases such as rheumatoid arthritis [10]. The mechanism of action of this drug is inhibition of DNA, RNA and protein synthesis by binding to DHFR enzyme which catalyzes the reduction of dihydrofolate (inactive state) to tetrahydrofolate (active state) which participates in activation of folate dependent enzymes required for DNA synthesis and methylation [11]. Methotrexate has been associated with declined fertility in mice which is seen as a result of aberrant sperm morphology i.e. abnormal head and tail which affects the sperm motility and contributes to decrease in the sperm count [12].

Amaranthus viridis also known as green amaranth is an annual herb 75-100 cm in height, numerous branches, the leaves are slender glabrous and ovate, having long petiole [13]. This herb has high nutritional value and is used for treating inflammation, boils and abscesses and purification of blood. The plant contains essential nutrients and has good levels of calcium, iron, beta carotene, folic acid and ascorbic acid [14]. The leaf extract of *Amaranthus viridis* has been used to study its anti-oxidative properties and the alkaloids were found to be pharmacologically active and potential promoter of oxidation [15-17]. A study done to evaluate nutritional and mineral compositions of green leafy vegetables in Assam (India) showed high levels of zinc content in *A. viridis* (6.50mg/100g) [18].

We conducted a mice study aimed at finding out whether increased folic acid intake or use of natural plant source such as *Amaranthus viridis* extract can be used as a dietary supplement to reduce or prevent sperm anomalies on undergoing treatment with anti-folate drug such as methotrexate which is known to cause sperm depletion and dampen the sperm motility due to irregular head and tail morphology conditions in mice.

MATERIALS AND METHODS

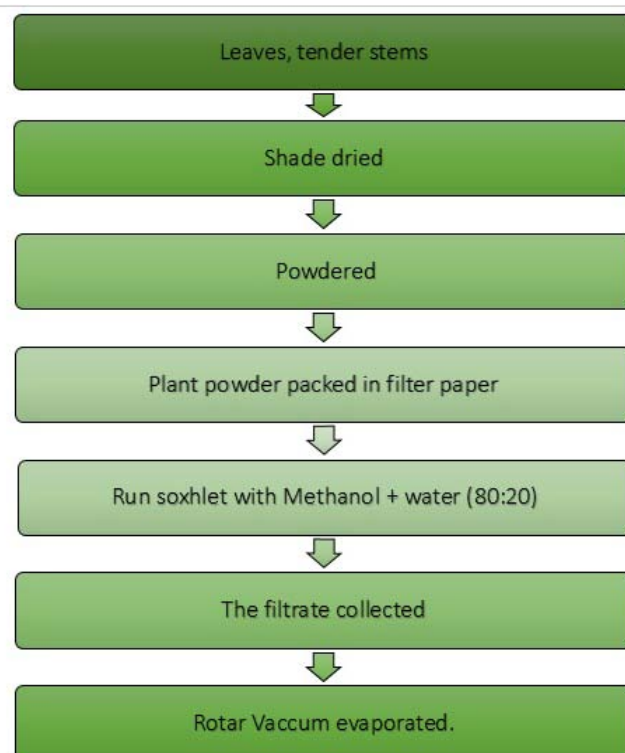
Ethics approval-The ethical standards of experiments were in accordance with the guidelines provided by the CPCSEA (Reg # 1754/PO/RC/S/14/CPCSEA).

STUDY DESIGN

Plant Extract

Amaranthus viridis (Figure 1) is commonly known as Thotakura in Telugu, India. *A. viridis* seed's were purchased from

local market, Ranga Reddy district, Telangana, India. The plants were grown in black soil with good agricultural practices. The plants were authenticated by T. Sudha Rani, Taxonomist-Sarojini Naidu Vanita Maha Vidyalaya in Hyderabad-India. After 30-45 days of vegetative growth, the stem and the leaves were used for extraction. The leaves were washed in distilled water for two or three times and shade dried. The dried leaves and the stem were coarsely crushed, powdered and stored in air tight container for further use. The sample was extracted using methanol (Hi Media) and water (80:20) with the help of Soxhlet apparatus in three replicates. The extract was further concentrated using the rotary vacuum evaporator under reduced pressure and stored at 4°C in a refrigerator.



In vitro studies

Healthy Albino mice (*Mus musculus*) around eight to ten (8-10) weeks old males, weighing 21 to 28 grams were procured and maintained in CPCSEA approved animal breeder, (Reg # 1754/PO/RC/S/14/CPCSEA) and housed in a clean room. The mice were kept at standard temperature of 22 °C (\pm 3 C) with 50% to 70% relative humidity, light and dark (12h) photoperiod was maintained. The feed used was commercially available pellets from Hindustan lever limited, Mumbai and autoclaved Milli Q water.

The mice were divided into four groups each with five animals. In each cage a minimum of two animals were kept.

Dose

Folic acid (Hi Media) 400µg per day is the daily requirement for teens and adults. In the experiment 250µg was given per dose.

Group-1: Methotrexate of 0.300mg/kg per mice, once a week for three weeks.



Figure 1 *Amaranthus viridis* plant.

Group-2: Methotrexate and folic acid 250µg per mice, once a week for three weeks.

Group-3: Methotrexate and *Amaranthus* Extract 250µg per mice, once a week for three weeks.

Group-4: The control animals were given distilled water 2ml per mice, once a week for three weeks.

The albino mice were treated with above vehicles and the animals were examined for clinical manifestations. The mice were sacrificed by cervical dislocation and the cauda epididymis was collected for sperms. From each group two mice were used, one mice was used for checking abnormalities and other for sperm count. The epididymis was minced in 0.7% saline, large fragments were removed and centrifuged for five minutes at 2000 rpm. Then the supernatant was obtained and sperm count was performed using Neubauer chamber. Similarly, one mouse was used from each group for checking sperm head abnormalities. After collecting the cauda epididymis it was minced in sodium citrate (2.2%) centrifuged at 1000 rpm for five minutes. Smears were prepared after staining in Eosin Y for observation under microscope (Figure 2).

The methotrexate in the present study induced changes in the morphology of the sperm. The shape of the sperm plays an important role in analyzing the morphological abnormalities.

Chemical composition	(Per 100 g of edible portion).
Water	88.9
Energy (Kcal)	18
Protein (mg)	3.5
Fat (g)	0.3
CHO (g)	0.3
Fibre (g)	2.6
Ca (mg)	270
Fe (mg)	130
P (mg)	65
Mg (mg)	3.0
Carotene µg	1725
Thiamine mg	0.07
Riboflavin mg	0.22
Niacin mg	0.7
Folic acid mg	85
Ascorbic acid mg	42

Sources: Holland et al., 1991; Leung et al., 1968

It has been observed that the sperm abnormalities occurred in MTX-treated mice. After three weeks of treatment there was some difference seen in the head morphology or the tail region. The head region varied in shapes and could be microcephalic or macrocephalic, pyriform, amorphous or elongated.

The MTX + folic acid treated animals mostly showed normal features of head shape and tail than MTX primed alone.

The MTX + *Amaranthus* extract treated animals showed a decrease in the head morphological deformations when compared to MTX alone.

Med Calc version 11.5.1.0 was used for performing Odds ratio test (i) to assess damage between Control and MTX treated groups and (ii) to assess the damage in groups supplemented with Folic Acid (FA) and *Amaranthus* Extract (AE) as compared to the MTX treated groups.

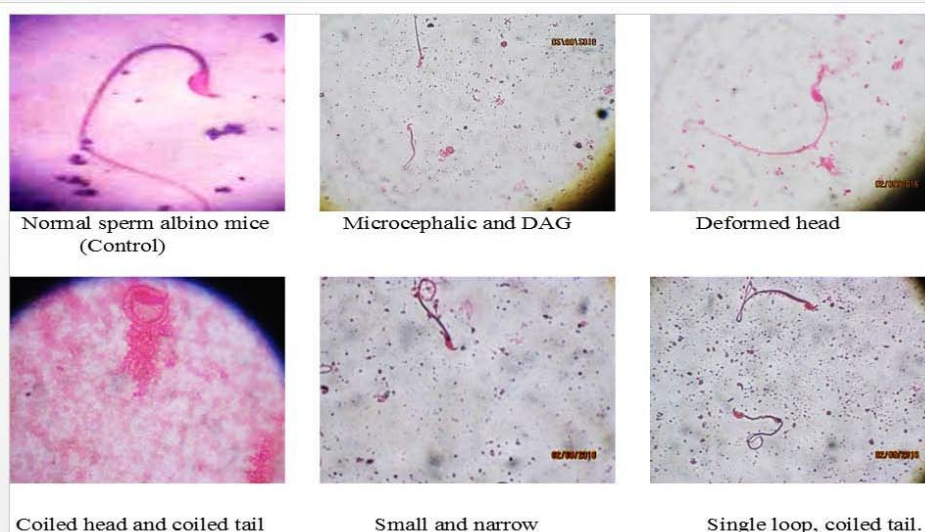


Figure 2 The abnormal sperms with head and tail defects in methotrexate treated mice.

- I. Damage induced by methotrexate on the sperm head and tail morphology is significant at $p=0.034$ when compared to the controls (Table 2 (a) and 2 (b)).
- II. Number of sperms with reduced motility ($p=0.0001$), dead sperms ($p=0.0003$) and abnormal head morphology ($p=0.015$) showing highly significant damaging effect of methotrexate on mice fertility Table 2 (c).
- III. The number of abnormal sperms decreased in FA and AE treated mice. Sperms in the AE extract and FA treated mice showed significantly improved mean values; FA and AE supplemented groups showed drastic improvement in motility ($p^{FA} = 0.0053$, $p^{AE} = 0.0024$), and reduction in number of dead sperms ($p^{FA} = 0.0059$ and $p^{AE} = 0.0092$), whereas it did not show a great impact on the head morphology ($p^{FA} 0.38$, $p^{AE} = 0.15$).
- IV. Effect of FA and AE on head morphology in methotrexate exposed cells: Reduced damage, Odds ratio not very significant at $p^{FA}=0.30$ and $p^{AE}=0.37$.

The abnormal sperms with head or tail defects are as shown in Figure 2.

DISCUSSION

According to Lock and Soares (1980) sperm evaluation is said to provide a quantitative measure for identifying genetic damage via estimating the count, morphology and motility in addition to other aspects of the seminal fluid [19]. Dietary corrections may be found beneficial in providing respite to individuals having unsuccessful attempts in conception. It has been found that dietary intake of vitamin C improved sperm count, vitamin E intake improved total progressively motile sperms, and beta-carotene uptake improved both sperm count and motility. *A. viridis*, a natural source of folate has become a point of interest because it could prevent or reverse sterility in men undergoing methotrexate treatment and also in the other cases with general folate insufficiency.

Methotrexate is an anti-metabolite agent that is used in treatment of neoplastic disorders and other ailments like rheumatoid arthritis etc. Exposed individuals reported a decrease in sperm count. Sussman and Leonard reported that the sperm

count often returned to normal after the drug was discontinued [20]. Shamberger et al., also reported Azoospermia in patients using MTX while studying the effects of postoperative adjuvant chemotherapy and radiotherapy on testicular function in men undergoing treatment for soft tissue Sarcoma [21]. A decrease in sperm count was reported by Van Scott & Reinertseon in men who underwent treatment of two weeks of intravenous MTX injection [22]. De Luca et al., however reported minimal to no suppression of spermatogenesis using the drug MTX [23].

Mouse is a mammalian model for studying the effect of drugs representing the total effects seen in the animal model with similar effects in humans. Anti-folates are used frequently in treatment of cancers and other neoplastic disorders, autoimmune disorders such as rheumatoid arthritis and have been under scrutiny due their impact on fertility in men. This concern has led to finding out ways of reducing and even reversing the impact of methotrexate and other similar kinds of drugs on spermatogenesis. This study aimed at finding out whether folic acid or *A. viridis* extract administered to mice exposed to methotrexate showed any improvement in the declining condition of germ cells during spermatogenesis. The control group provided comparison as to how the sperm count declined and morphology was affected when mice were administered methotrexate alone or in combination with either folic acid or along with *A. viridis* extract. Low mice sperm count and deformities were found to be significantly reduced in the mice being administered methotrexate along with a dose of folic acid and also in mice which were given methotrexate combined with *A. viridis* extract. When compared to the group administered methotrexate along with folate, the group given a combination of methotrexate and *A. viridis* extract showed better results, this could be attributed to the other nutrients and metabolites such as ascorbic acid and β -carotene present in the plant extract.

The results observed in mice could serve as information about whether dietary intake of folate and natural plant source could reduce the side of effects of drugs and medicines taken during the course of treatment and help prevent male sterility. Since *A. viridis* extract showed promising results, a study with higher dose and longer duration could help us find out if natural plant sources could be supplemented in the diet for reducing the overall impact of drugs on the spermatogenesis in mice. Studies

Table 2 (a): Damage induced by methotrexate on the sperm head and tail morphology.

Dose	No. of sperms examined	No. of abnormal Sperms	Mean %.	Amorphous	Pyriform	Elongate	Micro-cephalic	Macro-cephalic	Tail Abnormalities
Control	2000	90	4.5	40	-	-	18	23	09
MTX	2000	120	6	52	7	14	20	7	20
MTX+FA	2000	105	5.25	43	09	14	14	15	10
MTX+AE	2000	107	5.35	41	08	18	13	17	10

Table 2 (b): Damage induced by methotrexate on the sperm head and tail morphology is significant at $p=0.034$.

Dose	No. of sperms examined	No. of abnormal Sperms	Odds ratio (95% CI)	p value
Control	2000	90	-	-
MTX	2000	120	1.35 (1.02-1.79)	0.034
MTX +FA	2000	105	1.66 (0.66-1.13)	0.30
MTX+AE	2000	107	0.88 (0.67-1.15)	0.37

Table 2 (c): Sperm Motility, dead and abnormal sperms.

Groups	Sperm motility Mean%	Odds ratio 95% CI	P value	Dead sperms Mean %	Odds ratio 95% CI	P value	Sperm-head abnormalities Mean %	Odds ratio 95% CI	P value
Control	180/200= 90	-	-	14/200 = 7	-	-	8/200 = 4	-	-
MTX	140/200 =70	3.85 (2.22-6.66)	0.0001	40/200 = 20	3.32 (1.74-6.32)	0.0003	21/200 = 10.5	2.81(1.21-6.5)	0.015
MTX + FA	164/200 =82	0.51 (0.31-0.82)	0.0053	20/200 = 10	0.44 (0.24-0.79)	0.0059	16/200 = 8	0.74 (0.37-1.46)	0.38
MTX + AE	166/200 =83	0.47 (0.29-0.77)	0.0024	21/200 = 10.5	0.46 (0.26-0.82)	0.0092	13/200 = 6.5	0.59 (0.28-1.21)	0.15

conducted in mice could help us in finding out alternate therapies for reducing male sterility in men arising due to similar kind of drugs and involving similar metabolic pathways.

CONCLUSION

In humans the association between sperm quality and male infertility has been known now for forty years. Motility and morphology are excellent markers for evaluating sperm fertilizing capacity. However, abnormal sperm morphology may not be the only factor associated with recurrent spontaneous abortions. In the present work, dietary supplementation with FA and AE improved sperm motility and reduced dead sperm count to a highly significant extent however, it might be noted that the sperm head abnormalities in these mice improved only marginally as was the induction of damage (low). The study emphasizes that this kind of dietary supplementation would prove to be highly beneficial to sub-fertile men not subjected to MTX therapy.

A. viridis as a natural source of dietary supplementation of folate may be a good alternative to direct folic acid given in the form of tablets to individuals with folic acid deficiency due to various health and lifestyle issues. Folic acid supplements are routinely given to pregnant women; in addition to this, it might help to include these supplements in the male partner to resolve a good deal of infertility in couples with sperm anomalies as the primary cause for lack of conception and aid in improving chances for a healthy pregnancy in these couples.

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